DEVELOPMENT NEWER ANALYTICAL TECHNIQUES FOR THE ESTIMATION OF BENFOTIAMINE AND ITS COMBINATION WITH PYRIDOXAMINE DIHYDROCHLORIDE AND RESVERATROL

A Dissertation submitted to

THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY,

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Submitted by

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Coimbatore - 641 044.

APRIL 2017

CERTIFICATE

This is to certify that the dissertation entitled **"Development Newer Analytical Techniques for the Estimation of Benfotiamine and its Combination with Pyridoxamine dihydrochloride and Resveratrol"** being submitted to The Tamil Nadu Dr.M.G.R Medical University, Chennai was carried out by **GOBI. A** in the **Department of Pharmaceutical Analysis,** College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under the supervision and guidance of **Dr. M.Gandhimathi, M.Pharm., Ph.D.,** Associate Professor, Department of Pharmaceutical Analysis, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore.

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CONTENTS

LIST OF ABBREVATIONS

HPTLC	High Performance Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
ICH	International Conference on Harmonization
BEN	Benfotiamine
PYR	Pyridoxamine dihydrochloride
RES	Resveratrol
gm	Gram
M	Molar
mM	Millimolar
mg	Milligram
µL	Microliter
µg	Microgram
ng	Nanogram
min	Minute
R_t	Retention time
T_f	Tailing factor
R_s	Resolution
LOD	Limit of detection
LOQ	Limit of quantification
RSD	Relative standard deviation
UV	Ultra violet
KH_2PO_4	Potassium dihydrogen ortho phosphate

INTRODUCTION [1-12]

The analytical method is a generic process combining the power of the scientific method with the use of formol process to solve any type of research problem. It involves the steps like identify the problem to solve, choose an appropriate process, use the process to hypothesize analysis or solution elements, design an experiment to test the hypothesis perform the experiments to accept, reject or modify the hypothesis, check the performance before steps until the hypothesis is accepted, implement the solution continuously to improve process as opportunities arise.

The need of new analytical methods is vital in new drug development pharmaceutical products formulated with one or more than one drug was referred to be combination drug products, which are intended to meet previously unmet patient needs by combining the therapeutic effects of two or more drugs in one product. These combination products can produce daunting challenges to the analytical chemist responsible for the validation of analytical methods.

As a result of new drug research, number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities, development of patient resistance and introduction of better drugs to competitors, under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary to develop newer analytical method for drugs.

The information obtained from analytical research is used to identify potential sources of safety problems in the products. The analytical effort required to provide this information can be divided into a number of analytical tasks which are as below,

- ❖ Determination of identify, purity of starting materials and intermediates used in manufacturing the drug substance.
- ❖ Isolation and identification of trace impurities in drug substance.
- ❖ Determination of degradation rates and products.
- ❖ Establishment of an analytical reference standard for the drug substance.

The analytical methods require the meet crieterian like sensitivity, specificity, precise, robust and raggedness. The degrees of complexity acceptable in the analytical procedure vary considerably from task to task and therefore require a variety of analytical techniques to satisfy them.

Basic criteria for new method development of drug analysis:

- ➢ Drug or drug combination may not be official in pharmacopoeia.
- ➢ A proper analytical procedure for the drug may not be available in the literature.
- ➢ Analytical methods for the quantitation of the drug may not be available.
- ➢ Analytical methods for a drug in combination with other drugs only available.
- ➢ The existing analytical procedures may require expensive reagents and solvents.

Analysis of drugs by UV – Visible spectrophotometry

Analytical samples absorption in the UV-Visible region is often strong and therefore allows many substances to be measured in the low part per million ranges in non-absorbing media. Good sensitivity combined with ready availability of simple, accurate and inexpensive spectrophotometers have made UV- Visible spectrophotometry one of the most widely used instrumental techniques in organic analysis.

There are various spectrophotometric methods are available which can be used for the analysis of a samples.

- ✓ Simultaneous equation method
- ✓ Derivative spectrophotometric method
- ✓ Absorbance ratio method
- ✓ Difference spectrophotometry

Chromatography

Chromatography is the most powerful and versatile technique available to the modern analyst. A chromatographic procedure can separate a mixture of components into individual components and quantitatively determine the amount of each component present.

Development of chromatography has been quite remarkable in many ways. On one side the development of liquid chromatography was vens slow and arduous, particularly in the early years, whereas in comparison, the rate of development of gas chromatography was almost meteoric. HPTLC method for analyzing drug in single or multicomponent dosage forms required only primary knowledge, about the nature of sample namely, structure, polarity, volatility, stability and solubility parameters. An exact recipe for HPTLC, however also same like HPLC cannot be provided because method development involves considerable error and trial procedures.

The availability of disposable TLC plates, the problem caused by samples with high matrix contents, which block the HPLC columns and likely to give ghost peaks is eliminated by HPTLC system is automated and controlled throughout by software and is fully responsible and standardized cGMP - compliant technique.

It generates comparable analytical data with low level of uncertainly and is suitable for in process control for product analysis in simultaneous long-term comparison as batch-to-batch conformity for stability testing.

Stability indicating analysis method is employed for the analysis of sample stability in pharmaceutical industry. ICH guidelines explicity requires conduct of forced decomposition studies under a variety of conditions like pH, light, oxidation, dry heat and separation of drug from degradation products. Drugs containing functional groups such amides, esters, lactams, lactones easily undergo hydrolysis. Drug containing functional groups such as thiols, thioesters, easily undergo oxidation. Drug containing functional groups such as olefins, aryl halo derivatives, aryl acetic acids, aromatic nitro groups, N- oxide easily undergoes photo decomposition. This was the first step carried out in a drug structure to assess the likely decomposition route; secondly, collection of information on physicochemical properties was done which may be an easy exercise when the degradation products are known and available in the pure form.

HPLC systems can be modular basis in which the users can use different modules (e.g. Column and detector) depending on the analytical requirements. All basic compounds are difficult to be separated or purified on silica based reversed phase material for liquid chromatography because undesired adsorption of the basic nitrogen containing group to acidic sites on the silica surface.

The HPLC method developments focus specifically a suitable approach for isocratic reversed phase method development, since this is most commonly used in pharmaceutical field.

- Samples description includes the number of compounds: chemical structure/function, molecular weight, pKa, UV spectra, matrix, solubility and analyte concentration.

- Specification of goals includes analysis, purification or preparation? Qualitative and quantative analysis? Resolve all compounds and what is precision needed?
- Pretreatment of sample includes ready to inject or clean up needed? For example, dilution, buffering or solubility.
- Selection of detector where UV is the most desirable detector.
- The order of selection of method is,
  1st choices: RPLC, ION PAIR OR NPLC
  2st choices: IEC, SEC, HIC

Special cases: high molecular weight, isomers, inorganic, carbohydrates

- Initial runs can be isocratic and gradient.
- Optimization of resolution includes how much resolution needed? Time per run? Operating pressure? Solvent consumption and narrow peaks?
- Validate before the release of method which meets all goals, robust, routine method.

Validation as such cannot improve the process but it confirms and assures that the process has been well maintained and operates as it should validation is a basic requirement to ensure quality and reliability of the results for all analytical applications. It is essential to employ well characterized and fully validated analytical methods to yield reliable and recognized results in the laboratories while analyzing the registration batch and accelerated stability testing samples. It is also essential to emphasize that each analytical technique has its own characteristics, which will vary from analyte to analyte. The importance of validation is producing reliable, repeatable and accurate results for routine analysis and stability analysis studies carried out during the entire period of work. Validation of analytical procedures is directed to the four most common types of analytical procedures such as identification tests, quantitative test for impurity content, limit test for control of impurities and quantitative test for the active moiety in samples of drug substance or drug product.

Key parameters of the analytical method validation includes, precision (repeatability and reproducibility), linearity and range, limit of detection (LOD), limit of quantification (LOQ), selectivity/specificity, robustness, stability system suitability studies.

Statistics is a wide tool useful in all disciplines especially in research studies. One should know about the importance of statistical tools and how to use them in their research o survey. The quality assurance of the work must be dealt with the statistical operations to control and verify the analytical procedures as well as the resulting data making mistakes in analytical work is unavoidable. This is the reason why a multitude of different statistical tools is required some of them simple, some complicated and often very specific for certain purposes. In analytic work, the most important common operation is the comparison of data or sets of data, to quantity of accuracy and precision. Fortunately with a few simple convenient statistical tools most of the information needed in regular laboratory work can be obtained in t-test, F-test, ANOVA test and regression analysis. Clearly statistics are a tool not an aim. The value of statistics lies with organizing and simplifying data to permit some objective estimate showing that an analysis is under control or that a change has occurred.

REVIEW OF LITERATURE [13-18]

The need of survey of literature is to see what has and has not been investigated and identify data sources that other researchers have used. To learn how others have defined and measured key concepts and develop alternative research projects. Review of literature helps to demonstrate understanding and ability to critically evaluate research in the field and to provide evidence that may be used to support one's own findings.

A review of literature was conducted for the analysis of Benfotiamine, and its combination with Pyridoxamine dihydrochloride and Resveratrol are described below,

Siva sankari K.A (2016) et al., have developed and validated UV spectroscopic method for the estimation of Benfotiamine in bulk and solid dosage form. The simple, precision, accurate, UV spectrophotometric methods namely dual wavelength spectrophotometric method and difference UV spectrophotometric method have been developed using 0.1 N HCL as solvent. The HCL drug obeyed Beer's law in range of 5-25µg/ml. The overall percentage recovery found to be 99-100%.

B. Pavan aditya (2016) et al., have developed a new simple, precision, sensitive and validated RP-HPLC method for the estimation of Benfotiamine in bulk and pharmaceutical dosage form. The chromatographic conditions used for the separation was phenomenox Luna C_{18} (4.6×250mm.5µ) and mobile phase comprised of acetonitrile: methanol: water: 0.1% OPA (40: 20: 35:5 v/v). The flow rate was 1.0ml with detection at 249nm. The retention time was found to be 3.84 min. The linearity was found to be in the range of 5-35µg/ml for benfotiamine with correlation coefficient of 0.999. The method was successfully applied to pharmaceutical formulation.

Deepali A. Nanaware (2011) et al., have developed a simple, sensitive and rapid RP-HPLC method for the estimation of Benfotiamine and Metformin hydrochloride in pure and in pharmaceutical dosage forms. Thermo hypersil BDS-C_{18} column (250mm×4.6mm, 5µ Germany) with isocratic conditions was used with mobile phase containing mixture of methanol and aqueous phosphate buffer (10mM of potassium phosphate adjusted with 3.2 with OPA) in the ratio of 80: 20. The flow rate was 1ml/min and effluents were monitored at 239nm and eluted at 2,583 min (BEN) and 3.233 min (MET). Calibration curve was plotted with range of 1-6µg/ml for BEN and 0.1-5µg/ml.

S. Poongothai (2014) et al., have developed a simple, precise, rapid and validated selective RP-HPLC method for the simultaneous determination of Benfotiamine (B1) 100mg, Pyridoxine hydrochloride (B6) 100mg Mecolamine (B12)100mcg and Alpha-lipoic acid 100mg in Multivitamin capsules. The method was x- tetra reverse phase (RP-18, 250×4.6mm, 5µm) column and gradient elution. The aqueous mobile phase contained 0.05M phosphate buffer adjusted pH 2.5 and acetonitrile. Separation and quantification was achieved by changing the proportion of the system linearity. Detection was carried out in the range of 200 to 600nm using photodiode array detector set at 320 nm and further analysis was carried out using UV detector.

Marsza. M (2013) et al., have developed a rapid and sensitive HPLC method with coulometric electrochemical and UV detection for analysis of vitamin (B1) Thiamine, (B6) Pyridoxamine, Pyridoxal and Pyridoxine and (B12) in Animal and Plant foods has been developed. A combination of acid digestion and enzymatic extraction of release protein bound and a phosphorylated vitamin followed by HPLC analysis was applied. The analysis were carried out on a LC 18 column 5µ (250cm×4.6mm) using the mobile phase consisting of methanol: phosphate buffer (10:90) and 0.018M Triethylamine, adjusted pH 3.5 following 1.0ml/min. The method offer excellent linearity with regression coefficient $r > 0.998$.

Roopa.S. Pai (2013) et al., have developed a new formulation of t-Rvt loaded PLGA nanoparticle with potential stearth properties was prepared by nano precipitation method in our laboratory. The desired chromatographic separation was achieved on a phenomenox C_{18} column under isocratic conditions using UV detection as 306nm. The optimized mobile phase consisted of a mixture of methanol: 10mM potassium dihydrogen phosphate (pH 6.8): Acetonitrile (63:30:7 v/v/v) at a flow rate of 1.0ml/min. The linear regression analysis for the calibration curves showed a good linear correlation over the concentration range 0.025-2.0µg/ml with determination co-efficient exceeding 0.9997.

AIM AND OBJECTIVE

Benfotiamine is an antioxidant dietary supplement drug used in diabetic neuropathy. The US FDA approved benfotiamine in January 18, 2006 to diabetic neuropathy and benfotiamine in combination of pyridoxamine dihydrochloride and resveratrol.

Literature reveals very few analytical techniques for the estimation of benfotiamine from tablets. Also there is no analytical method reported for the combination of benfotiamine with pyridoxamine dihydrochloride and resveratrol, till date.

Hence the major objective of the present research work is,

- To develop and validate UV spectroscopic method for the estimation of benfotiamine in bulk drug and tablet formulation.

- To develop and validate stability indicating HPTLC method for the determination of benfotiamine and its application of accelerated stability studies of benfotiamine tablets.

- To develop and validate a HPTLC method for estimation of benfotiamine in combination with pyridoxamine dihydrochloride and resveratrol in combined dosage from.

- To develop a validated RP-HPLC method for estimation of benfotiamine in combination with pyridoxamine dihydrochloride and resveratrol tablet formulation.

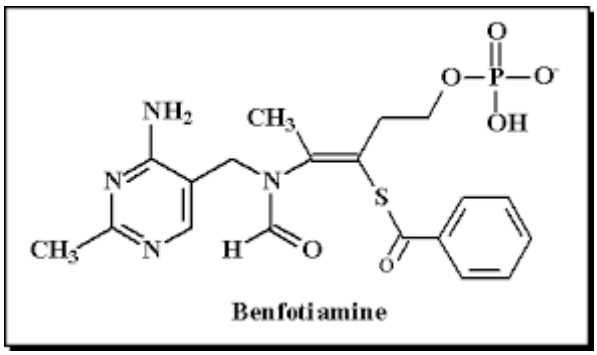
DRUG PROFILE[13,14,16,18]

Name : Benfotiamine

IUPAC Name : S-[2-{[(4-amino-2-methylpyrimidin-5-yl) methyl] (Formyl) amino}-5-(phosphonooxy) pent-2-en-3yl] Benzenecarbothioate.

Empirical Formula : $C_{19}H_{23}N_4O_6PS$.

Structure Formula :



Benfotiamine

Molecular Weight : 466.448 g/mol.

Description : White to off white powder.

Solubility : water, aqueous solvents.

Melting point : 165ºC

Bioavailability : 33.8%

Storage : Stored in tightly closed container in a drug & ventilated place.

Category : Vitamin B1

Action and use : Vitamin B1 analog. Synthetic S-acyl derivative of Thiamine, antioxidant, advanced glycation end Product supplement, treating diabetic complications.

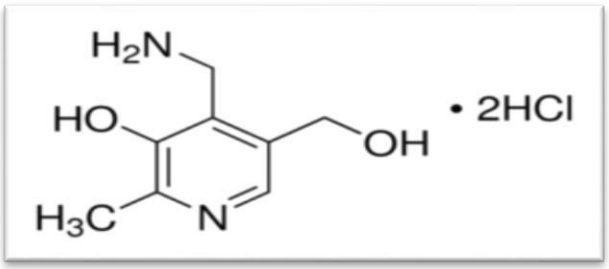
Available dosage forms: Tablets, Capsule.

Name : Pyridoxamine Dihydrochloride.

IUPAC Name : 4-(amino methyl)-5-hydroxy-6-methyl-3-pyridine Methanol dihydrochloride.

Empirical Formula : $C_8H_{14}CI_2N_2O_2$

Structural Formula :



Molecular Weight : 241.11 g/mol.

Description : Yellow to green powder.

Solubility : Water, DMSO, Methanol, Ethanol, Acetone.

Melting point : 226- 227ºC

Bioavailability : 65%

Storage : Stored in tightly closed container in a drug & ventilated place.

Category : Vitamin B6.

Action and use : An inhibitor of advanced glycation reactions, also Inhibits advanced lip oxidation reactions.

Dietary supplement and prevent the progression of Diabetic nephropathy.

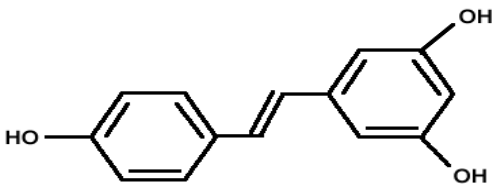
Available dosage forms : Tablet.

Name : Resveratrol

IUPAC Name : 3, 5, 4'-trihydroxy-Trans –stilbene.

Empirical Formula : $C_{14}H_{12}O_3$

Structural Formula :



Resveratrol

Molecular Weight : 228.25g/mol.

Descriptions : White powder with slight yellow cast.

Solubility : Water, DMSO, Ethanol, Methanol, Acetone.

Melting point : 261-263ºC

Bioavailability : 70%

Storage : Stored in a dry and cleaned place.

Category : Antioxidant.

Action and use : NF-Kb inhibitory action of resveratrol a probable Mechanism of neuroprotection in Diabetic neuropathy revolves around oxidative Stress, AGE formation, lipid peroxidation.

Available dosage form : Tablet.

Benfotiamine is available as single and in combination with pyridoxamine dihydrochloride & resveratrol as shown in below,

BRANDS AVAILABLE

Brand Name	Composition	Company
BENALGIS tab	Benfotiamine 75mg/100mg	FRANCO
FOTIA tab	Benfotiamine 150mg	BESTOCHEM
BENFAGE tab	Benfotiamine 100mg/200mg	ORCHID
BENFORCE tab	Benfotiamine 150mg	SHIELD
VITAMER-EA cap	Benfotiamine 50mg	CARSYON
AGELESS tab	Benfotiamine 50mg, pyridoxamine dihydrochloride 25mg, resveratrol 25mg	APEX
ASIA tab	Benfotiamine 50mg, pyridoxamine dihydrochloride 25mg, resveratrol 25mg	APEX

MATERIALS AND INSTRUMENTS

CHEMICALS AND SOLVENTS USED

- Water for HPLC
- Methanol HPLC grade (99.8%)
- Methanol AR grade (99.0%)
- Acetonitrile HPLC grade (99.9%)
- Triethylamine AR grade (99.0%)
- Petroleum ether AR grade (99.0%)
- Acetone AR grade (99.5%)
- Tetra butyl ammonium bromide
- Potassium dihydrogen orthophosphate
- Glacial acetic acid (99.0%)
- Orthophosphoric acid
- Distilled water

All the above chemicals and solvents were supplied by S.D fine chemicals Ltd, India, qualigens fine chemicals Ltd, Mumbai, India, sigma – Aldrich chemicals Pvt. Ltd., Maharashtra, India.

MATERIALS USED

- Pre- coated silica gel $60F_{254}$ on aluminum sheets were procured from Merck, Germany.
- Cellulose filters 0.45µm.
- Poly tetra fluoro ethylene filter 0.45µm, dimension 47mm.
- LICHRO. SPHER ®100 RP-18e (5µ), (250×4.6MM column).
- Whattmann filter paper

INSTRUMENTS

- ✓ Shimadzu digital electronic balance
- ✓ Elico Pvt, Limited, india, pH meter.
- ✓ Camag HPTLC system (with TLC scanner-3) wins CATS software and Linomat 5 as application device.
- ✓ Shimadzu HPLC system with SPD-M10 A VP system PDA with 20µl fixed volume manual injector and LC-MS solution software.
- ✓ Jasco V-630 spectrophotometer with a pair of 1 cm quartz cuvette.
- ✓ Sonicator (leelasonic ultrasonic Sonicator)

I. DEVELOPMENT AND VALIDATION OF UV SPECTROSCOPIC METHOD FOR THE ESTIMATION OF BENFOTIAMINE IN BULK DRUG AND TABLET FORMULATION

Benfotiamine is having chromosphores in its structure and hence it will absorb electromagnetic radiation between 200-400nm. This character is used for estimating Benfotiamine by UV spectroscopic method.

OPTIMIZATION OF EXPERIMENTAL CONDITION:

Selection of solvent:

The solubility of drug was checked in different solvents and found to be completely soluble in methanol. A UV spectrum of drug solution in methanol was recorded. Smooth spectrum with reasonable absorbance was obtained and hence it was selected as solvent for further studies.

Preparation of stock solution:

Stock solution Benfotiamine (1000µg/ml) was prepared LR grade 99% methanol in 10ml glass volumetric flask.

Selection of wavelength:

The working standard solutions were scanned in UV range of (200-400nm) using a Jasco V-630 UV-Visible spectrophotometer with cells of 10mm length against the same solvent used as blank. It was scanned in the range of 200-400 nm and it shows absorbance maxima at a maximum wavelength of 240nm.

VALIDATION OF THE METHOD

The developed method was validated as per ICH guidelines [19] in terms linearity, accuracy, precision, repeatability, LOD, LOQ and robustness.

Linearity and range:

Various aliquots were prepared from the secondary stock solution (100µg/ml) concentration ranging from 4-24µg/ml. The samples were scanned in UV-Visible spectrophotometer against methanol as blank. At 240nm the absorbance of standards were measured. The calibration graph was plotted against concentration versus absorbance. The slope, intercept and correlation coefficient values were calculated.

Accuracy:

Solutions were prepared in at levels 50% and 100% of test concentration using Benfotiamine working standard as per the test method and taken absorbance of each solution in triplicate. The Recovery results showed that the proposed method has an acceptable level of accuracy.

Precision:

Precision of the method was demonstrated by intra-day, inter-day and repeatability variation studies.

Precision of the method was studied by

- Intra-day precision
- Inter-day precision
- Repeatability

Intra-day precision:

In intra-day variation study nine different solutions of same concentration 12µg/ml and 16µg/ml were analyzed three times in a day i.e. morning, afternoon, and evening and the absorbance is noted and from the mean, standard deviation and RSD% were calculated.

Inter-day precision:

In inter-day variation studies, solution of same concentration 12µg/ml and 16µg/ml were analyzed three times for the consecutive days and the absorbance result mean, standard deviation and RSD% was calculated.

Repeatability:

The repeatability was studied by carrying out the analysis of the standard drug concentration 12µg/ml in the linearity range of the drug for six times on the same day and the response for each concentration was recorded and %RSD was calculated.

Limit of detection (LOD) and limit of quantification (LOQ):

The lowest concentration detected (LOD) and lowest concentration quantified (LOQ) were estimated from the set of five calibration curves used to determine method linearity.

$$LOD = 3.3\times\sigma/S \text{ and } LOQ = 10\times\sigma/S$$

Where, σ = the standard deviation of y intercepts of regression lines

S = the slope of the calibration curve

Solution stability:

The stock solution of Benfotiamine was stored at room temperature and under refrigeration. The absorbance of solution was measured at different time interval. The % reductions in absorbance were noted to calculate stability of solution.

Analysis of formulation:

Benalgis (100mg) was assayed by developed UV spectrophotometric method. Twenty tablets were weighed and finely pulverized and the quantity of homogenized powder equivalent to 10mg of Benfotiamine was transferred to 10ml volumetric flask. It was dissolved by adding small volume of methanol and sonicated for five minutes. The volume was made up with the same solvent. The solution was filtered using whattmann filter paper. Suitable dilution was made and the corresponding UV spectrum of formulation solution was recorded. The absorbance was noted at 240nm and the amount per tablet and % label claim were calculated.

II. DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR THE DETERMINATION OF BENFOTIAMINE AND ITS APPLICATION TO ACCELERATED STABILITY STUDIES OF BENFOTIAMINE TABLETS

The present work is to develop an economic accurate, reproducible and stability indicating HPTLC method for the determination of benfotiamine in the presence of its degradation products and related impurities from pharmaceutical formulation.

Selection of plate

A pre-coated silica gel G_{60} F_{254} coated on aluminum sheet was selected for the study.

Selection of solvent

Drug should be soluble in the solvent used.

Drug should show stability in the solvent used.

Solvent should be volatile.

Ideal properties of a solvent employed for HPTLC are,

Methanol was selected as the solvent here for further studies as the benfotiamine was soluble and fulfilling above criteria.

Selection of wavelength

The sensitivity of HPTLC method depends upon the proper selection of wavelength for UV detection. An ideal wavelength is the one that gives maximum absorbance and good response for the drug at the lower concentration is to be selected. The UV spectrum of Benfotiamine was recorded in HPTLC scanner and maximum wavelength was found to be 275nm. This wavelength selected for the study.

Detection of optimum mobile phase

A mobile phase system that would give dense compact spots and good separation from solvent and application position was to be selected. Initially, different solvent systems were tried and there observations were as given below:

Table 1: Selection of Mobile Phase

Solvent system tried	Observation
100% Methanol	Drug move but spot not clear.
100% Acetic acid	Drug move but spot not clear.
Acetic acid: Methanol(1:9v/v)	Drug retained. tailed spot
Acetic acid: Methanol(2:8v/v)	Drug retained, tailed spot
Acetic acid: Methanol: Tetra butyl ammonium bromide(9:1v/v:2 drops)	Drug retained, tailed spot
Acetic acid: Methanol: Triethylamine(8:2v/v: 2drops)	Drug retained, tailed spot
Acetic acid: Methanol: Triethylamine(9:1v/v: 2drops)	**Good separation with compact spot.**

Chromatographic development procedure:

Suitable volume of standard solution was spotted in the form of bands having a band width of 6mm on precoated silica gel G_{60} F_{254} HPTLC plate, 8mm from the bottom and 15mm from the side edges in the form of bands. Linear ascending development was carried out and the optimized mobile phase consisted of glacial acetic acid: methanol: Triethylamine (9:1v/v: 2 drops). The optimized chamber saturation time before chromatographic development was 15 min and the length of chromatographic run was 8cm. Subsequent to the development, HPTLC plates were dried in a current of air with help of an air dryer. Densitometric scanning was performed and all measurements were made in the reflectance absorbance mode at 275nm, with slit dimension (5.00 × 0.45mm)

the source of radiation was deuterium lamp emitting a continuous UV spectrum between 200-400nm. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light and evaluation was done by ordinary linear regression analysis.

Optimization of chamber saturation

The above fixed mobile phase was added to one side of twin trough chamber previously rinsed with acetone and dried then saturation times fixed as 15 minutes.

Fixed Experimental Conditions

Stationary phase	:	Pre-coated silica gel $G_{60}F_{254}$ on aluminum sheets
Mobile phase	:	Glacial acetic acid: Methanol: Triethylamine
Chamber saturation time	:	15 min
Plate migration distance	:	80mm
Band width	:	6mm
Slit dimension	:	5 × 0.45mm
Source of radiation	:	Deuterium lamp
R_F value	:	0.29±0.02
Detection wavelength	:	275nm

Preparation of stock solutions

10mg of Benfotiamine were taken into a 10ml standard flask and made up to the volume with methanol to get a stock solution of 1000µg/ml.

VALIDATION OF THE METHOD:

The developed method was validated as per ICH guidelines. The validated method was carried out in terms of linearity, accuracy, precision, repeatability, LOD and LOQ.

Linearity and range:

A solution containing 1000µg/ml of Benfotiamine was prepared in methanol. Different volumes from 0.2 – 1.2µl of this solution were applied on the plate. After development the plate was scanned and peak area were noted. Linear regression data showed good correlation coefficient over a concentration range of 200-1200ng/spot.

Accuracy:

Recovery study was in order to ensure the suitability and reliability of HPTLC method, recovery studies were carried out. It was done by mixing known quantities of the standard drug (50 & 100% level) with the analyzed sample formulation and the contents were reanalyzed by proposed method.

Precision:

Precision of the method was studied by

- Intra-day precision
- Inter-day precision
- Repeatability precision

Repeatability of sample measurement

Repeatability of sample application

Intra-day precision:

Intra-day precision was studied by carrying out the analysis of the standard drug for two different concentrations for three times on the same day and %RSD was calculated.

Inter-day precision:

Inter-day precision was studied by carrying out the analysis of the standard drug for two different concentrations for three different days over a period of one week and %RSD was calculated.

Repeatability:

Repeatability of sample measurement:

Repeatability of measurement was determined by spotting 600ng/spot of drug solution on a pre-coated TLC plate and developed the plate and scanned six times %RSD was calculated.

Repeatability of sample application:

Repeatability of sample application was carried by spotting 4µl of drug solution is about six times on pre-coated TLC plate followed by development of plate and %RSD was calculated.

Limit of detection (LOD) and limit of quantification (LOQ):

The lowest concentration detected (LOD) and lowest concentration quantified (LOQ) were estimated from the set of five calibration curves used to determine method linearity.

$$LOD = 3.3\times\sigma/S \text{ and } LOQ = 10\times\sigma/S$$

Where, σ = the standard deviation of y intercepts of regression lines

S = the slope of the calibration curve

Robustness:

The robustness of the method is its ability to remain unaffected by small changes in practical conditions. Here the effect of change in conditions such as ratio of mobile phase (±0.1ml) and (±0.2min) were studied to prove robustness.

Specificity:

The peak purity of Benfotiamine was assessed by comparing its respective spectra at peak start, peak apex and peak end positions of the spot.

Stability studies:

When the developed chromatographic plate is exposed to atmosphere, the analytes are likely to be decomposed. It is necessary to study the stability of drug on TLC plate. It was studied by scanning the plate at different time interval and peak areas were compared with the peak area of freshly scanned plate. The developed TLC plate was found to be stable for about 12 hours for Benfotiamine.

ANALYSIS OF TABLET FORMULATION

To determine the amount of Benfotiamine in various tablet dosage forms (label claim 100mg per tablet), the contents of 20 tablets were weighed, their mean weight was determined, and they are finely powdered. An accurately weighed powder sample equivalent to 10mg of Benfotiamine was transferred into a 100ml volumetric flask containing 10ml methanol, followed by sonication for 10min. The resulting solution was filtered through whattmann filter. A volume of 0.6µl of the filtered solution (600ng/band) was applied on the HPTLC plate followed by development and scanning as per optimized chromatographic conditions.

Application of HPTLC method to Accelerated stability study of benfotiamine

Forced degradation studies:

To evaluate the stability indicating property of the developed HPTLC method, standard drug was subjected to acid/base hydrolysis, and oxidation. 100mg of pure drug Benfotiamine was accurately weighed and transferred to 100ml volumetric flask and diluted with methanol to obtain a final concentration of 1000µg/ml. The solution was further subjected to following forced degradation study.

Acid- induced degradation study:

To 15ml of the above methanolic standard drug solution 10ml of 1N, 0.1N, and 0.01N hydrochloric acid was added in three different 25ml volumetric flask and this solution was refluxed at 40ºC for 5 hrs. Neutralized solution was directly applied to HPTLC plate followed by development and scanning under optimized chromatographic conditions.

Base – induced degradation study:

To 15ml of the above methanolic standard drug solution 10ml of 1N, 0.1N, and 0.01N sodium hydroxide was added in three different 25ml volumetric flask and this solution was refluxed at 40ºC for 5 hrs. Neutralized solution was directly applied to HPTLC plate followed by development and scanning under optimized chromatographic conditions.

Hydrogen peroxide – induced degradation study:

To 15ml of the above methanolic standard drug solution 10ml of 30% hydrogen peroxide solution was added in 25ml volumetric flask and this solution was refluxed at 40ºC for 5 hrs. Neutralized solution was directly applied to HPTLC plate followed by development and scanning under optimized chromatographic conditions.

Degradation of placebo of formulation:

A placebo (or dummy pill) [20] is an inert substance, typically a tablet, capsule, or other dose form that does not contain an active drug ingredient. For example, placebo pills or liquids may contain starch, sugar, or saline.

The fixed chromatographic condition was applied to estimate placebo ingredients. The placebo was subjected for acid/base hydrolysis and oxidation as per the procedure monitored in degradation study. The placebo was prepared using excipients as below.

Excipients	Weight taken
Lactose	0.45g
Calcium carbonate	0.25g
Magnesium stearate	0.01g
Gelatin	0.24g
Poly vinyl pyrrolidine	0.05g

The placebo mixture was weighed dissolved and subjected to various stress conditions a volume of 0.6µl was spotted on TLC plate in triplicate analyzed using fixed chromatographic conditions described earlier.

Degradation of Benfotiamine in formulation:

The fixed chromatographic condition was applied to estimate Benfotiamine from tablets. The Benfotiamine tablet was subjected for acid hydrolysis, base hydrolysis, oxidative degradation, thermal degradation and photolytic degradation same as that bulk drug.

From the various stress samples prepared 0.6µl was spotted on TLC plate in triplicates analyzed using fixed chromatographic conditions described earlier. The chromatograms were obtained for any addition peak and change in benfotiamine peak.

III. DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR ESTIMATION OF BENFOTIAMINE IN COMBINATION WITH PYRIDOXAMINE DIHYDROCHLORIDE AND RESVERATROL IN COMBINED DOSAGE FORM.

The experimental work consists of optimization of chromatographic conditions development and validation of HPTLC method of Benfotiamine in combination of Pyridoxamine dihydrochloride and Resveratrol.

Selection of plate

A pre-coated silica gel G_{60} F_{254} coated on aluminum sheet was selected for the study.

Selection of solvent

Drug should be soluble in the solvent used.

Drug should show stability in the solvent used.

Solvent should be volatile.

Ideal properties of a solvent employed for HPTLC are,

Methanol was selected as the solvent of sample for further studies, as the drug was soluble and fulfilling above criteria.

Selection of wavelength

The sensitivity of HPTLC method depends upon the proper selection of wavelength for UV detection. An ideal wavelength is that the overlay that give maximum absorbance and good response for the drug to be selected at the lower concentration is to be selected. The UV spectrum of Benfotiamine, pyridoxamine and resveratrol was recorded in HPTLC scanner and maximum wavelength was found to be 285nm. This wavelength was selected for the study.

Detection of optimum mobile phase

A mobile phase system that would give dense compact spots and good separation from solvent and application position was to be selected. Initially, different solvent systems were tried and there observations were as given below:

Table 1: SELECTION OF MOBILE PHASE

Solvent system tried	Observation
Methanol: Ethanol: Chloroform: Acetone(1:1:5:3v:v:v:v)	Drugs move along with solvent front.
Methanol: Ethanol: Ether: Acetone(0.5:0.5:6:3v:v:v:v)	Pyridoxamine dihydrochloride and resveratrol spot separated and benfotiamine did not moved from starting position.
Methanol: Ethanol: Ether: Acetone: water(0.5:0.5:6:3v:v:v:2drops)	Pyridoxamine dihydrochloride and resveratrol spot separated and benfotiamine did not moved from starting position.
Methanol: Ether: Acetone: water(0.5:6.5:3v:v:v:2 drops)	Pyridoxamine dihydrochloride and resveratrol spot separated and benfotiamine did not moved from starting position.
Methanol: Ether: Acetone(0.5:6.5:3v:v:v)	Drugs move along with solvent front
Tetrahydrofuran: Chloroform: Isopropyl alcohol(1:8:1v:v:v)	Pyridoxamine dihydrochloride and resveratrol move along with solvent front and benfotiamine did not moved from starting position.
Methanol: Ethanol: Ether: Acetone: Glacial acetic acid(0.5:0.5:5.5:3:0.5v:v:v:v:v)	Pyridoxamine dihydrochloride and resveratrol move along with solvent front and benfotiamine did not moved from starting position.
Methanol: Ethanol: Ether: Acetone: Ammonia(0.5:0.5:6:3v:v:v:v:2 drops)	Pyridoxamine dihydrochloride and resveratrol move along with solvent front and benfotiamine did not moved from starting position.
Methanol: Ethanol: Ether: Acetone: Triethylamine(0.5:0.5:6:3v:v:v:v:2 drops)	Pyridoxamine dihydrochloride and resveratrol move along with solvent front and benfotiamine did not moved from starting position.

Solvent system tried	Observation
Glacial Acetic acid: Ether: Acetone: Triethylamine(7:2:1v:v:v:2 drops)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(8:1:1v:v:v:2 drops)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(6:2:2v:v:v:2 drops)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(6:3:1v:v:v:2 drops)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(5:4:1:v:v:v:2 drops)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(6:3:1v:v:v:4 drops)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(5:4:1v:v:v:2 drops)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(5.5:3:1:0.5v:v:v:v)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(5:3:1:1v:v:v:v)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(5.5:2:2:0.5v:v:v:v)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(5.5:1:3:0.5v:v:v:v)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(6:2:2v:v:v:4 drops)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(6:1.5:2.5v:v:v:4 drops)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(5.5:1.5:2.5:0.5v:v:v:v)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(5.5:1.5:2.6:0.4v:v:v:v)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(4:2:3.5:0.5v:v:v:v)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(4:1:4.5:0.5v:v:v:v)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(4:0.5:5:0.5v:v:v:v)	Drugs retained, tailed spot
Glacial Acetic acid: Ether: Acetone: Triethylamine(5.5:1.5:3v:v:v:5	Drugs retained, tailed spot

Solvent system tried	Observation
drops)	
Glacial Acetic acid: Ether: Acetone: Triethylamine: Tetra butyl ammonium bromide(5.5:1.5:3v:v:v:5 drops:2 drops)	**Good separation all three drugs with compact spot.**

Separation using glacial Acetic acid: Ether: Acetone: Triethylamine: Tetra butyl ammonium bromide

Different ratios of Glacial acetic acid: Ether: Acetone: Triethylamine: Tetra butyl ammonium bromide was tried, from which the ratio of (5.5:1.5:3v/v:5drops:2drops) was selected because it gave good separation with compact spots and three times check mobile phase the same separation in UV chamber. The peaks are symmetric with acceptable R_f value.

Optimization of chamber saturation

The above fixed mobile phase was added to one side of twin trough chamber previously rinsed with acetone and dried then saturation times from 15 minutes.

Fixed Experimental Conditions

Stationary phase : Pre-coated silica gel $G_{60}F_{254}$ on aluminum sheets

Mobile phase : Glacial acetic acid: Ether: Acetone: Triethylamine: Tetra butyl Ammonium bromide

Chamber saturation time : 15 min

Plate migration distance : 80mm

Band width : 6mm

Slit dimension	:	5 × 0.45mm
Source of radiation	:	Deuterium lamp
R_f value	:	0.12, 0.33, 0.86±0.02
Detection wavelength	:	285nm

Preparation of stock solutions

Stock solutions prepared in ratio (2:1:1) of Benfotiamine (200µg/ml), Pyridoxamine dihydrochloride (100µg/ml) and Resveratrol (100µg/ml) was prepared with methanol in 10ml volumetric flask.

VALIDATION OF THE METHOD:

The developed method was validated as per ICH guidelines. The validated method was carried out in terms of linearity, accuracy, precision, repeatability, LOD and LOQ.

Linearity and range:

The linearity of response for the stock solution was assessed using different volume of stock solution. Via., 1, 2, 3, 4, 5, 6µl were spotted on TLC plate to obtain the concentration of 100-600ng/spot. The spots were developed and evaluated densitometrically using CAMAG HPTLC system. Peak area were noted for each spot and plotted against concentration to get a linear graph.

Accuracy:

Accuracy of method was ascertained by performing recovery at two concentration level of 50% and 100% by spiking standard solution (400ng and 600ng) to the dosage form (600ng/band). A recovery study was performed in triplicate.

Precision:

Precision of the method was studied by

- Intra-day precision
- Inter-day precision
- Repeatability precision

  Repeatability of sample measurement

  Repeatability of sample application

Intra-day precision:

Intra-day precision was studied by carrying out the analysis of the standard drug of two different concentrations for three times on the same day and %RSD was calculated.

Inter-day precision:

Inter-day precision was studied by carrying out the analysis of the standard drug of two different concentrations for three different days over a period of one week and %RSD was calculated.

Repeatability:

Repeatability of sample measurement:

Repeatability of measurement was determined by spotting 600ng/spot of drug solution on a pre-coated TLC plate and developed the plate and scanned six times and %RSD was calculated.

Repeatability of sample application:

Repeatability of sample application was carried by spotting 6 times of 4µl of drug solution on pre-coated TLC plate followed by development of plate and %RSD was calculated.

Limit of detection (LOD) and limit of quantification (LOQ):

The lowest concentration detected (LOD) and lowest concentration quantified (LOQ) were estimated from the set of five calibration curves used to determine method linearity.

LOD = 3.3×σ/S and LOQ = 10×σ/S

Where, σ = the standard deviation of y intercepts of regression lines

S = the slope of the calibration curve

Robustness:

The effect of deliberate variations on method parameters like the composition of the mobile phase saturation time, development distance spot scanning time interval, wavelength scan time from spotting to chromatography and mobile phase volume was evaluated.

Specificity:

The peak purity of Benfotiamine was assessed by comparing its respective spectra at the three different levels, that is, peak start (S), peak apex (M) and peak end (E) positions of the spot.

Stability studies:

When the developed chromatographic plate is exposed to atmosphere, the analytes are likely to decomposed. It is necessary to study the stability of drug on plate. It was studied by scanning the plate at different time interval and peak areas were compared with the peak area of freshly scanned plate. The developed plate was found to be stable for about 24 hours for benfotiamine.

ANALYSIS OF TABLET FORMULATION

To determine the amount of Benfotiamine, Pyridoxamine dihydrochloride and Resveratrol in tablet dosage form (label claim 50, 25, 25mg per tablet respectively) 20 tablets were weighed, their mean weight was determined, and they were finely powdered. An accurately weighed powder sample equivalent to 10mg of Pyridoxamine dihydrochloride, Resveratrol and 20mg of Benfotiamine was transferred into a 10ml volumetric flask then added 5ml methanol, followed by sonication for 10 min and further dilution up to the mark with methanol. The resulting solution was filtered through whattmann filter paper and three microliters of the filtered solution (600ng/spot) was applied on the TLC plate followed by development and scanning as per optimized chromatographic conditions.

IV. DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF BENFOTIAMINE IN COMBINATION WITH PYRIDOXAMINE DIHYDRO-CHLORIDE AND RESVERATROL TABLET FORMULATION

Chromatographic method for separation:

Since the drug Benfotiamine, Pyridoxamine dihydrochloride and Resveratrol is polar in nature, RP-HPLC method was selected as separation technique.

Selection of solvent:

The drugs are soluble in methanol and also showed good stability. Hence methanol was selected as the solvent.

Selection of wavelength:

Good analytical separation can be obtained only by careful selection of wavelength for the detection. This choice requires knowledge of the UV spectrum of the sample component. A UV spectrum of Benfotiamine, Pyridoxamine dihydrochloride and Resveratrol was recorded in methanol and the overlays have shown good maximum absorbance at 254nm for all three drugs and it was selected for the study.

OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

Optimization of mobile phase:

Optimization of mobile phase was carried out in order to obtain ideal peak of benfotiamine, pyridoxamine dihydrochloride and resveratrol.

The mobile phase system consisting of potassium dihydrogen phosphate (KH_2PO_4) and methanol and acetonitrile was optimized for their ratio and pH of the buffer.

Effect of mobile phase ratio:

The effect of ratio was studied by varying strength such as given below,

<table>
<tr><th colspan="2">Mobile phase solvent</th><th colspan="2">Ratio</th></tr>
<tr><td colspan="2">Binary pumps used mobile phases</td><td rowspan="2">10: 90, 20: 80 & 40: 60</td><td rowspan="2">50: 50, 20: 80 & 30: 70</td></tr>
<tr><td>Buffer: Acetonitrile % v/v</td><td>Buffer: Methanol % v/v</td></tr>
<tr><td colspan="2">Isocratic pumps used mobile phases Buffer: Methanol: Acetonitrile % v/v</td><td colspan="2">50: 25+25, 50: 35+15, 60: 40, 65: 17.5+17.5 & 70: 15+15</td></tr>
</table>

Effect of pH:

Keeping the ratio of mobile phase constant (70:15+15 % v/v), the chromatograms were recorded at different pH between 3- 5.20. A good resolution and symmetrical peak was obtained at a pH of 4.90 and selected for further studies.

FIXED CHROMATOGRAPHIC CONDITIONS

Chromatographic method	:	RP-HPLC
Column (stationary phase)	:	LichroCart®250-4 column
Mobile phase	:	10mM KH2PO4: Methanol + Resveratrol
Ratio of mobile phase	:	70: 15 + 15v/v
Detection of wavelength	:	254nm
Flow rate	:	1.0ml/min
Retention time	:	2.20, 4.28 and 20.68± 0.02min.
Temperature	:	Room temperature

Chromatographic development procedure:

The HPLC system was stabilized for 40min, by passing mobile phase and detector was set as 254nm and flow rate of 1.0ml/min was maintained to get a stable baseline. One blank followed by six replicate of a single standard solution were injected to check the system suitability.

VALIDATION OF THE METHOD

The developed HPLC method was validated according to ICH guidelines in terms of specificity, linearity, accuracy, precision, robustness and LOD and LOQ.

Specificity:

The specificity of the method towards the drug was established through study of resolution factor of the drug peak from the nearest resolving peak. The peak purity of BEN, PYR, and RES was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E). Effect of excipients of formulation was studied for whether it interfered with the assay.

Linearity and range:

The mixed standard stock solution (200µg/ml of BEN, 100µg/ml of PYR and RES) was further diluted to get BEN, PYR and RES concentration in the range of 10-50µg/ml and 5-25µg/ml respectively. Linearity of the method was studied by injecting six concentration of the drug prepared in the mobile phase in triplicate into the LC system keeping the injection volume constant, The peak areas were plotted against the corresponding concentration to obtain the calibration graphs.

Accuracy:

Accuracy of the method was carried out by applying the method to drug sample (BEN, PYR and RES combination tablet) to which known amount of BEN, PYR and RES standard powder corresponding to 50 and 100% of label claim had been added (standard addition method) mixed and the powder was extracted and analyzed by running chromatogram in optimized mobile phase.

Precision:

The precision of the method was verified by intraday, interday and repeatability precision studies.

- Intra-day precision
- Inter-day precision
- Repeatability

Intra-day precision:

Intraday precision was determined by injecting standard solutions in between linearity range (20, 30µg/ml for BEN and 10, 15µg/ml for PYR and RES) were injected three times on the same day and % RSD was calculated.

Inter-day precision:

Inter-day precision was determined by injecting standard solutions in between linearity range (20, 30µg/ml for BEN and 10, 15µg/ml for PYR and RES) were injected for three days and % RSD was calculated.

Repeatability:

Repeatability studies were performed by analysis of different concentration 10µg/ml of BEN and 5µg/ml of PYR and RES six times on the same day and %RSD was calculated.

Limit of detection (LOD) and Limit of quantitation (LOQ):

Limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ respectively.

LOD is the lowest concentration of the analyte that can produce a response detectable above the noise level of the system.

S/N = 2/1 or 3/1

LOQ is the lowest of the analyte that can be accurately and precisely measured.

S/N = 10/1

Robustness of method:

To evaluate robustness of a HPLC method, few parameters were deliberately varied. The parameters included variation of flow rate, percentage of methanol in the mobile phase and solvents. The response factors like retention time, resolution, asymmetric factor for these changed conditions were noted.

Stability of solution:

The standard solution of benfotiamine, pyridoxamine and resveratrol kept under room temperature. It was injected periodically. Stability was studied by looking for any change in retention time, resolution and peak shape, when compared to chromatogram of freshly prepared solution. The solution was stable 24hrs under room temperature.

System suitability parameters:

The system suitability parameters like peak area, tailing factor, theoretical plate count, and resolution and retention time were calculated from the standard chromatograms.

Analysis of a marketed formulation:

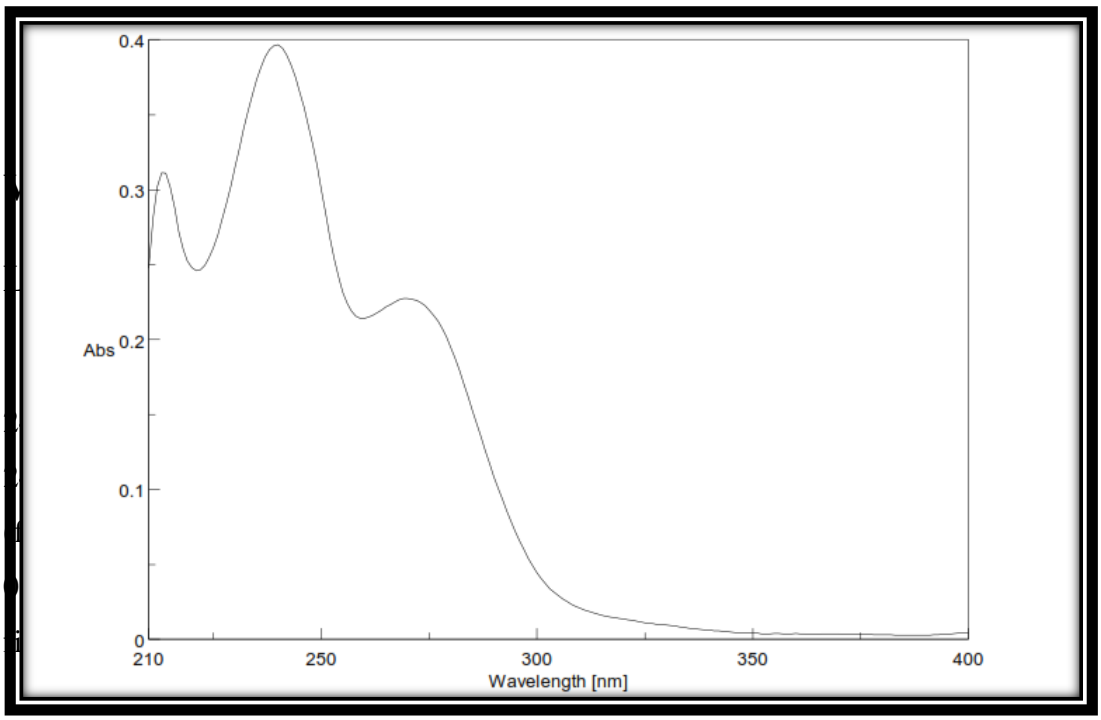
To determine the content of BEN, PYR and RES in conventional tablet (brand name: Ageless, label claim 50mg of BEN, 25mg of PYR and RES per tablet) twenty tablets were weight, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 20mg of BEN and 10mg of PYR and RES was transferred into a 10ml volumetric flask containing 6ml methanol, sonicated for 5min and diluted up to 10ml with methanol. The sample solution was then filtered using 0.45µm filter. The above stock solution was further diluted to get sample solution of 20µg/ml BEN and 10µg/ml PYR and RES respectively. A 20µl volume of sample solution was injected into HPLC, six times, under the conditions described above. The peak areas were measured at 254nm.

RESULTS AND DISCUSSION

I. DEVELOPMENT AND VALIDATION OF UV SPECTROSCOPIC METHOD FOR THE ESTIMATION OF BENFOTIAMINE IN BULK DRUG AND TABLET FORMULATION

The stock solution containing 1000µg/ml of Benfotiamine was diluted using methanol to get a concentration of solution 10µg/ml. The resulting solution was scanned in the UV spectrometer in the range of 200-400nm. The maximum absorbance was found to be at 240nm. The conjugated chromophore system of Benfotiamine shown smooth and it is shown fig 1.

Fig 1: UV spectrum of Benfotiamine in methanol



VALIDATION PARAMETERS

Linearity and range:

A calibration graph concentration between range of Benfotiamine (2-24µg/ml) and its absorbance shown in fig, 2. The calibration data table shown in table 1. The slope, intercept and correlation coefficient values were 0.034, 0.0016 and 0.9990, respectively. The overlay spectrum of all concentration shown in fig,3.

Fig 2: Calibration graph of Benfotiamine (4-24µg/ml)

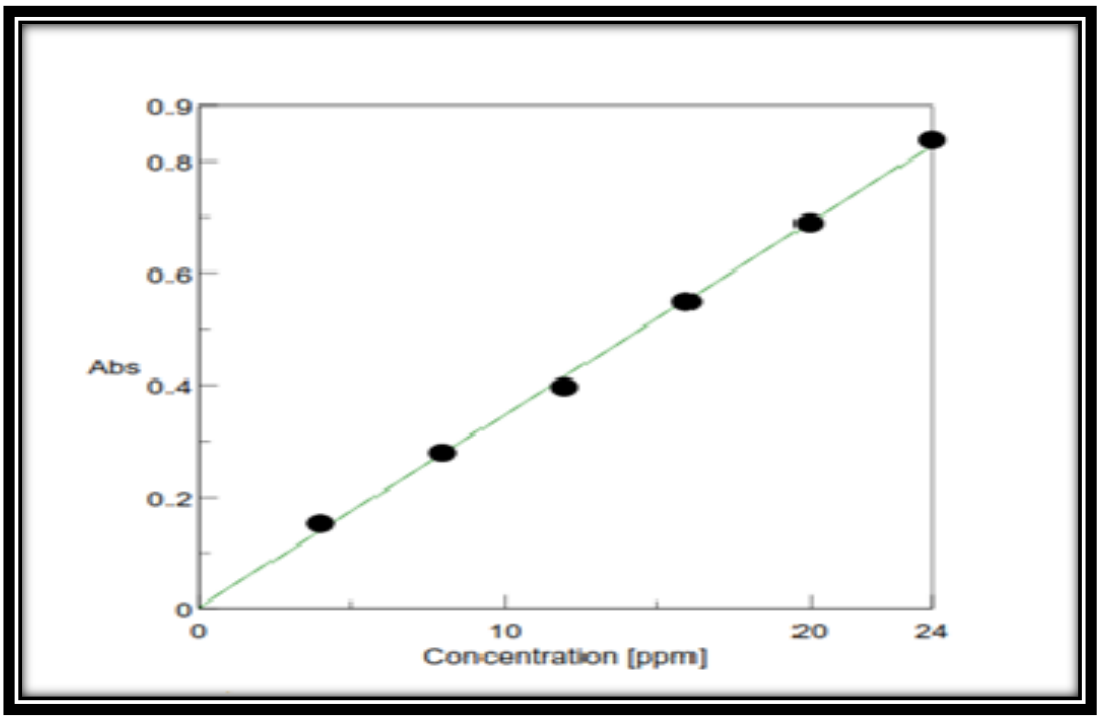
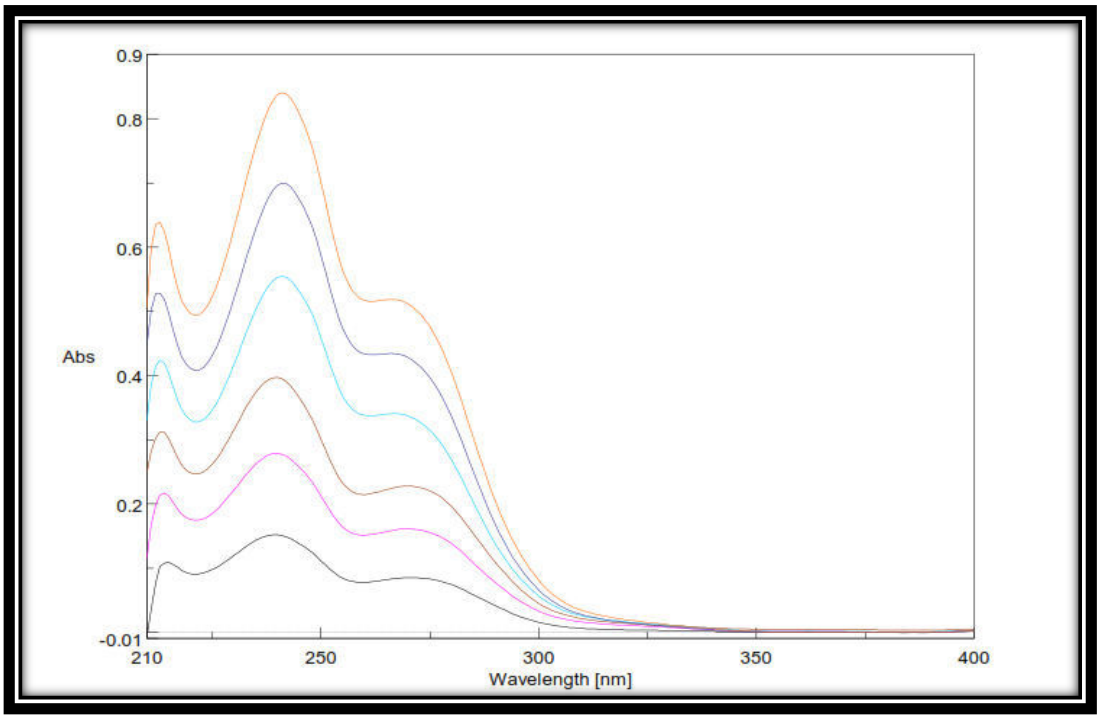


Table 1: Calibration table for Benfotiamine (4-24µg/ml)

Concentration (µg/ml)	Absorbance
4	0.1512
8	0.2776
12	0.3959
16	0.5474
20	0.6884
24	0.8368

Fig 3: Overlay spectrum of Benfotiamine



Precision:

The method precision was obtained by determining the assay by preparing six sample of selected concentration its 12 & 16µg/ml of Benfotiamine. The method precisions for intraday, interday, repeatability were studied and %RSD are shown in table 2,3&4, respectively.

Table 2: Intra-day precision

Concentration(µg/ml)	Absorbance	%RSD
12	0.4010	0.29
	0.4026	
	0.4035	
16	0.5458	0.21
	0.5468	
	0.5482	

Table 3: Inter-day precision

Concentration(µg/ml)	Absorbance	%RSD
12	0.4018	0.09
	0.4026	
	0.4022	
16	0.5678	0.08
	0.5687	
	0.5678	

Table 4: Repeatability

Concentration(µg/ml)	Absorbance	%RSD
12	0.4010	0.19
	0.4026	
	0.4035	
	0.4018	
	0.4026	
	0.4022	

Solution stability:

The working standard solutions of Benfotiamine were stored at room temperature and refrigerated. They were analyzed at different time interval. From the absorbance noted it was seen that the solution is stable up to 24hrs at room temperature (table 5). The solution stored under refrigeration was found to be stable up to 30hrs.

Table 5: Solution stability

Concentration(µg/ml)	Time(hours)	Absorbance
12	0	0.4010
	3	0.4026
	6	0.4035
	12	0.3959
	24	0.3902
	30	0.3752

ANALYSIS OF FORMULATION:

The solution of formulation was prepared and UV spectra was recorded and shown in fig 4. The amount present per tablet, % label claim, and %RSD were calculated and presented in table 6. The result obtained by UV spectroscopic method for Benfotiamine is satisfiable and there was no interference due to excipients.

Fig 4: UV spectrum of Benfotiamine formulation

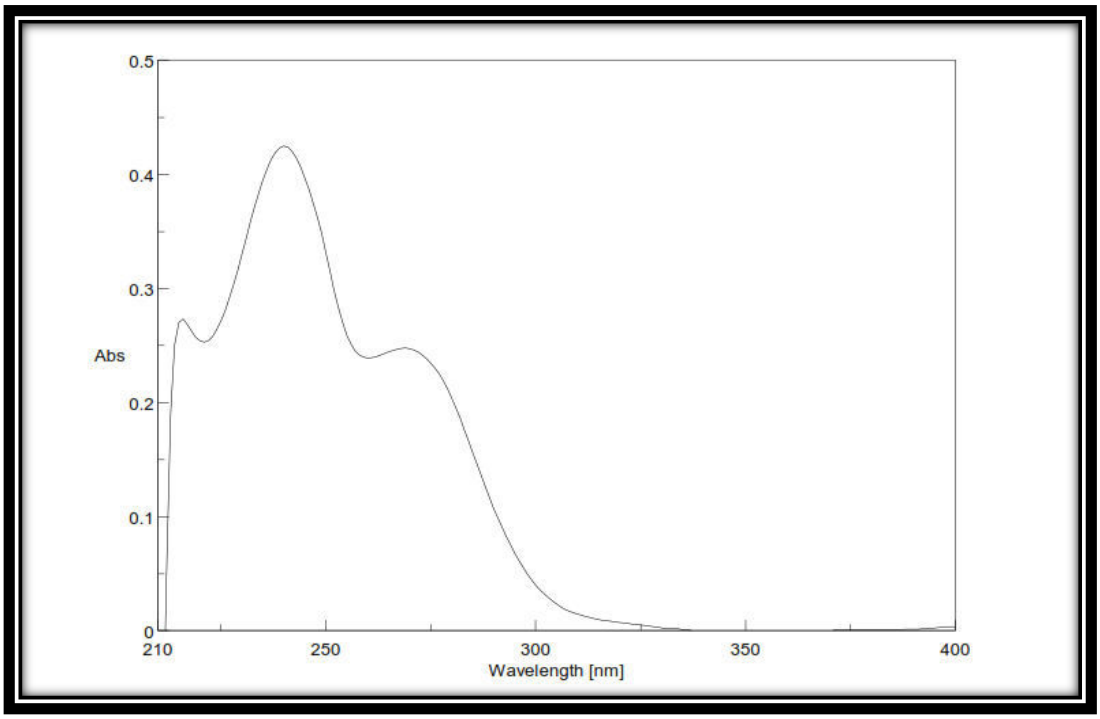


Table 6: Results of analysis of formulation

Trade name	Amount of drug/ tab		% label claim	% RSD	SEM
Benalgis	Labeled	Found	99	0.87	0.03
	100mg	99mg			

***Average of six observation**

Recovery study:

Recovery studies were carried out at 50% and 100% levels. The percentage recovery and percentage RSD of the results were calculated and shown in table 7. They were found to be good.

Table 7: Recovery study

Level	% recovery	% RSD	SEM
50%	96.54%	0.35	0.16
100%	98.87%	0.69	0.22

***Average of six observation**

II. DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR THE DETERMINATION OF BENFOTIAMINE AND ITS APPLICATION OF ACCELERATED STABILITY STUDIES OF BENFOTIAMINE TABLETS

Method development and optimization for stability indicating HPTLC method

Different mobile phase compositions are tried for development and validation of benfotiamine by stability indicating HPTLC method. Among these mobile phase systems, **Glacial acetic acid: Methanol: Triethylamine (9:1 %v/v: 2drops)** was selected because in this system compact spot and dense spot with good separation were obtained.

METHOD VALIDATION

The results of validation of the stability indicating method of Benfotiamine using Glacial acetic acid: Methanol: Triethylamine (9:1%v/v: 2drops) as the mobile phase are described below.

Linearity and range

Linear regression data showed a good correlation coefficient over a concentration range of 200-1200ng/spot. The calibration data are shown in table 8, and the slope, intercept and correlation coefficient values were found are presented in table 9. The calibration graph is shown fig 5. The standard spectrum and chromatograms obtained at different concentrations of Benfotiamine are shown in fig 6-12.

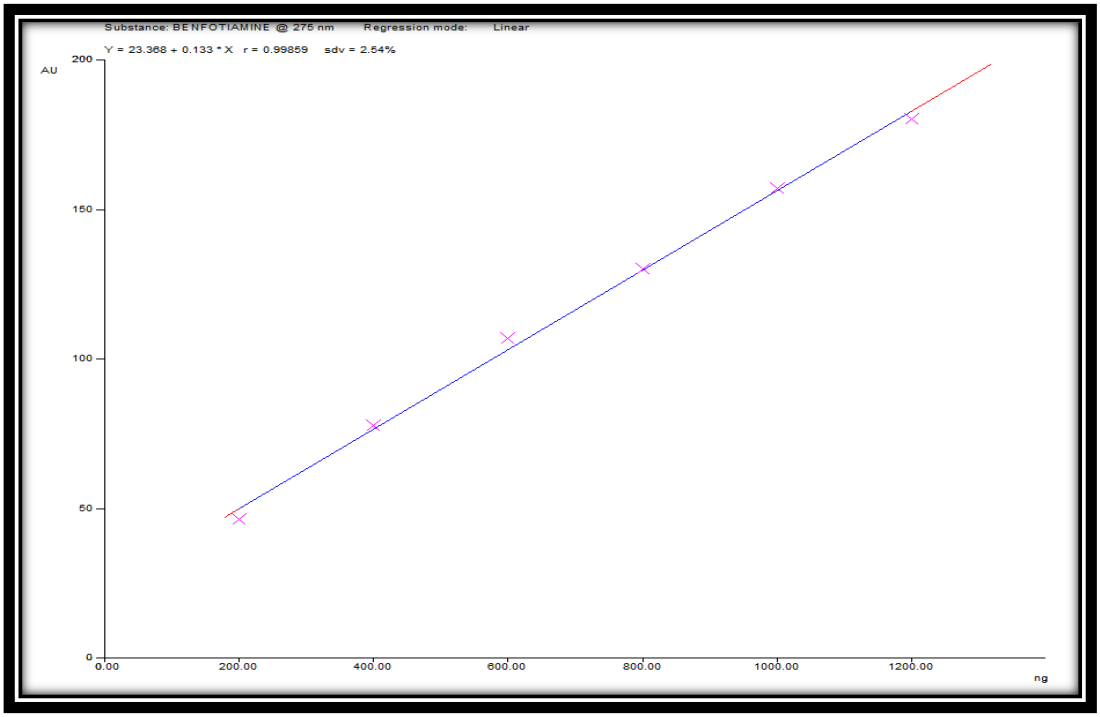
Table 8: Calibration data for Benfotiamine

Concentration (ng/spot)	Peak area
200	1628.2
400	2733.0
600	3658.4
800	4562.3
1000	5367.3
1200	6176.0

Table 9: Slope, intercept & correlation coefficient values

Slope	23.368
Intercept	-0.133
Correlation coefficient	0.998

Fig 5: Calibration graph of benfotiamine (200-1200µg/ml)



Standard spectrum and chromatograms

Fig 6: Spectrum of Benfotiamine

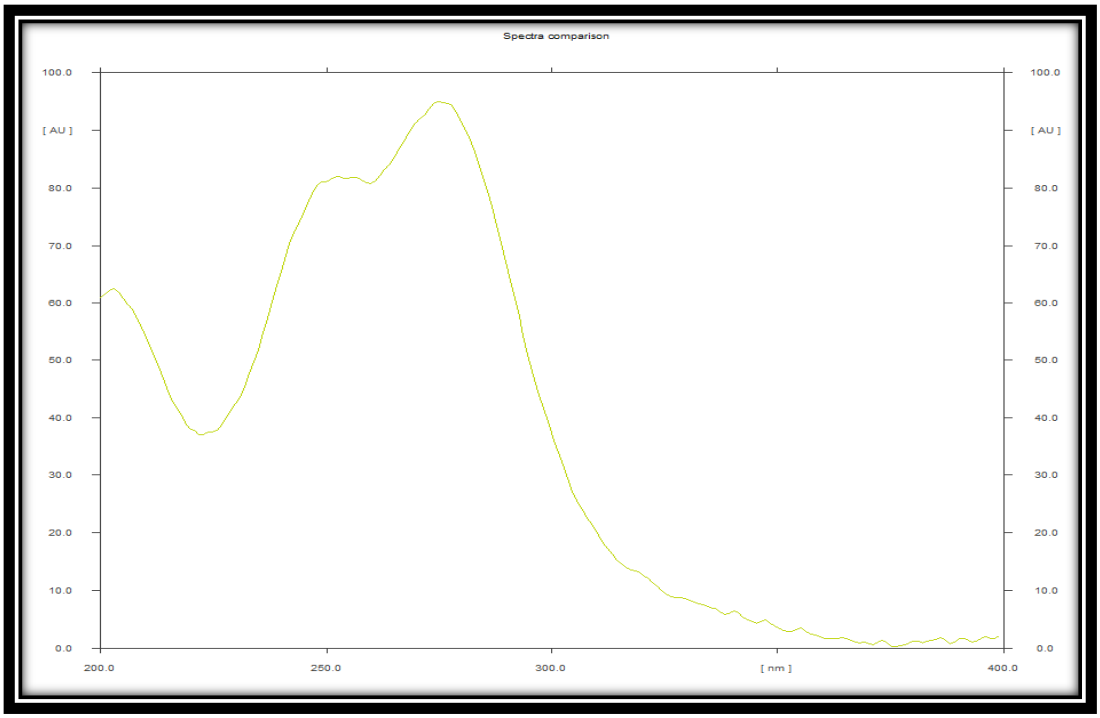


Fig 7: Benfotiamine 200ng/spot

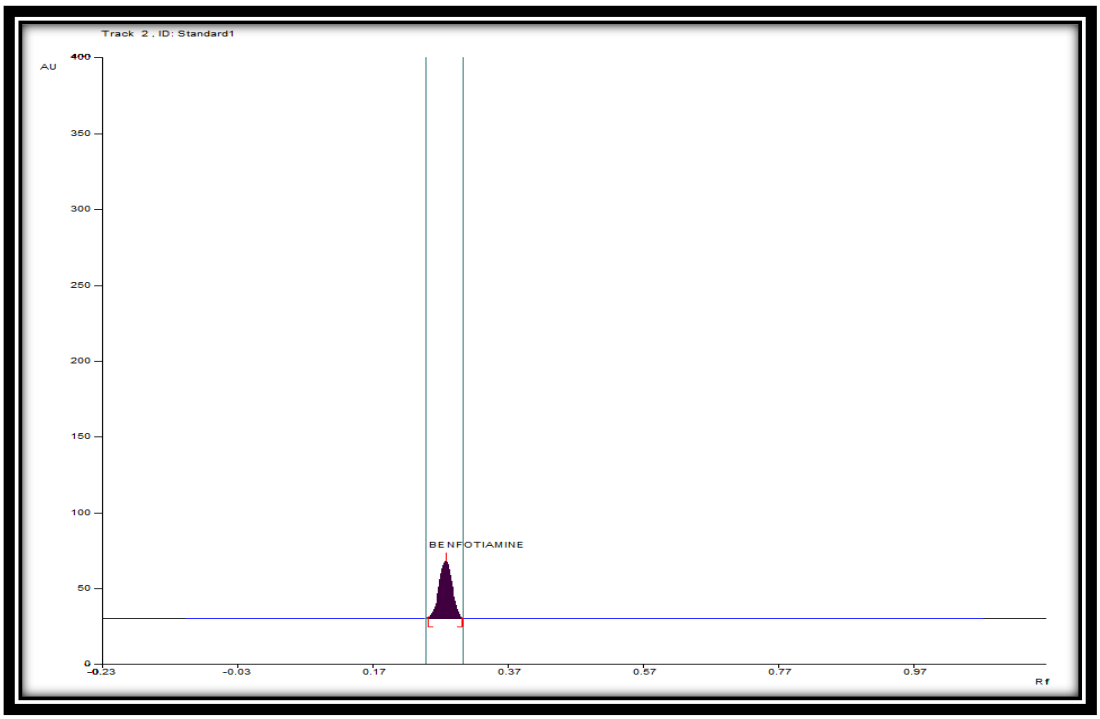


Fig 8: Benfotiamine 400ng/spot

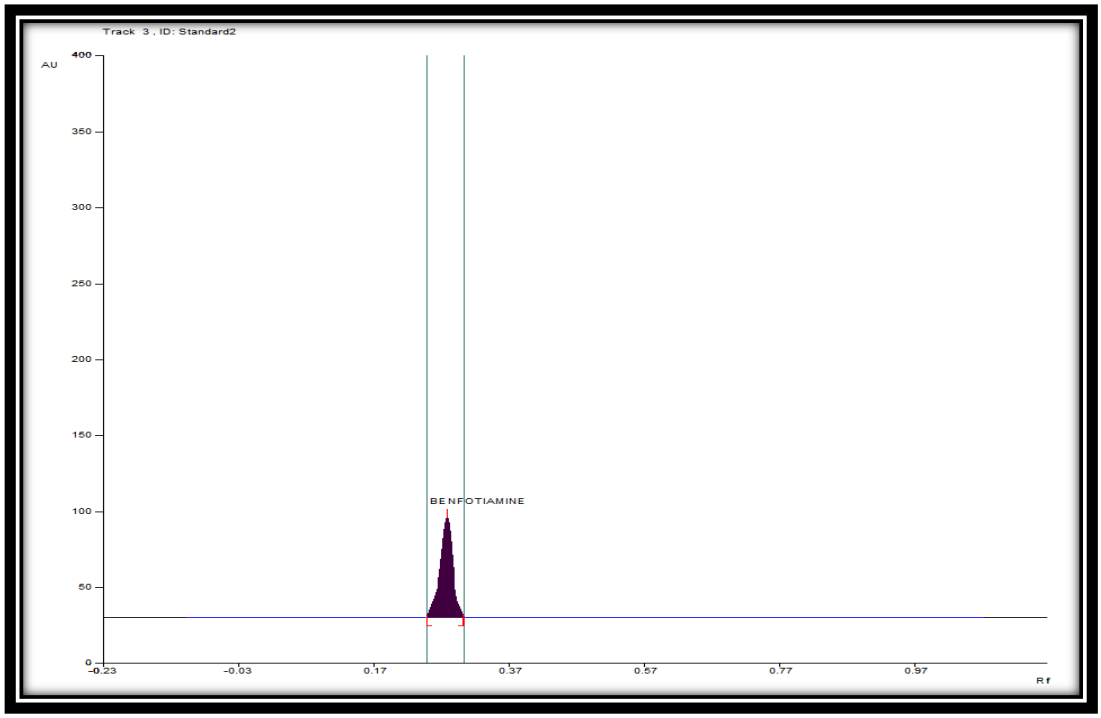


Fig 9: Benfotiamine 600ng/spot

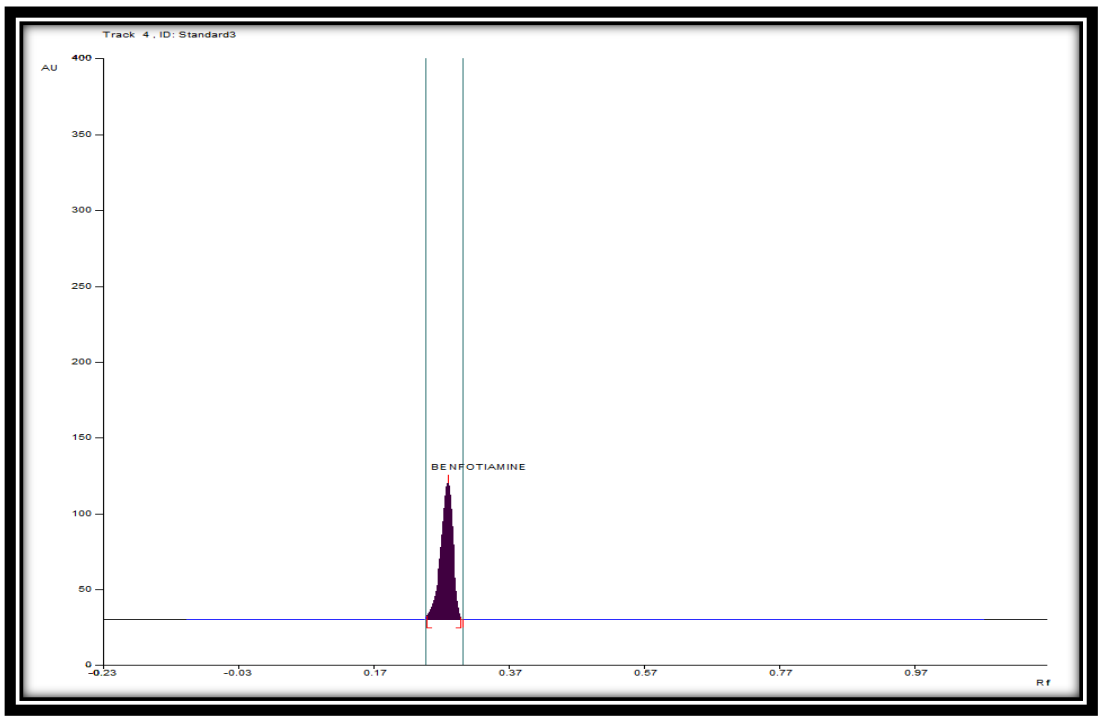


Fig 10: Benfotiamine 800ng/spot

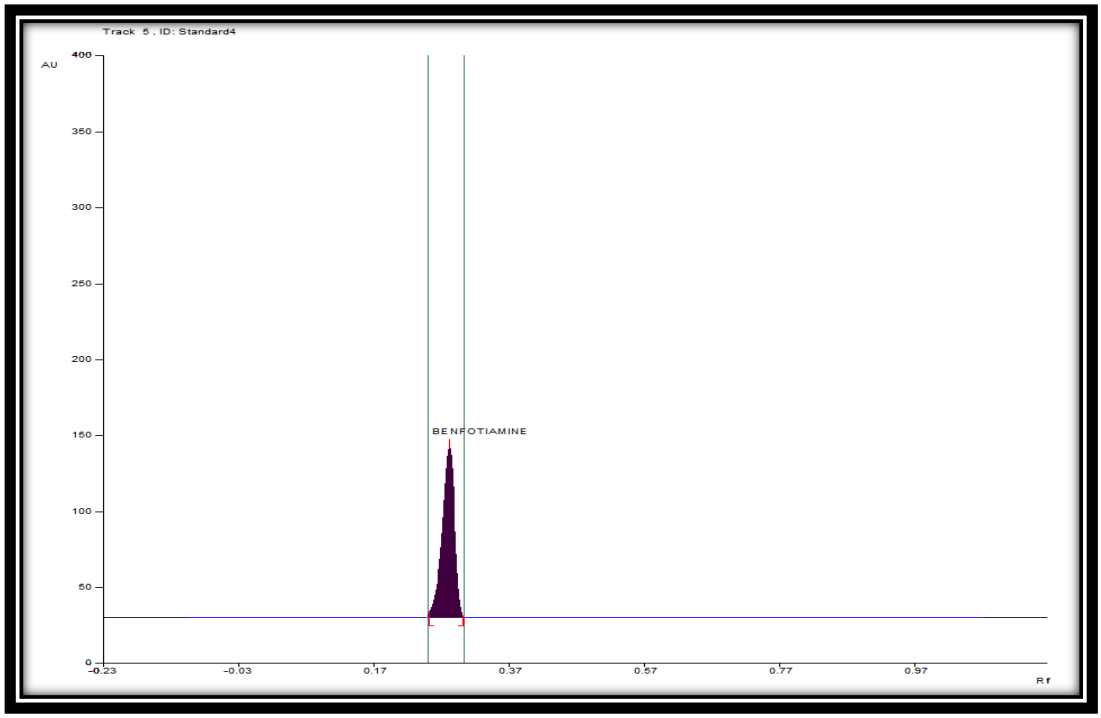


Fig 11: Benfotiamine 1000ng/spot

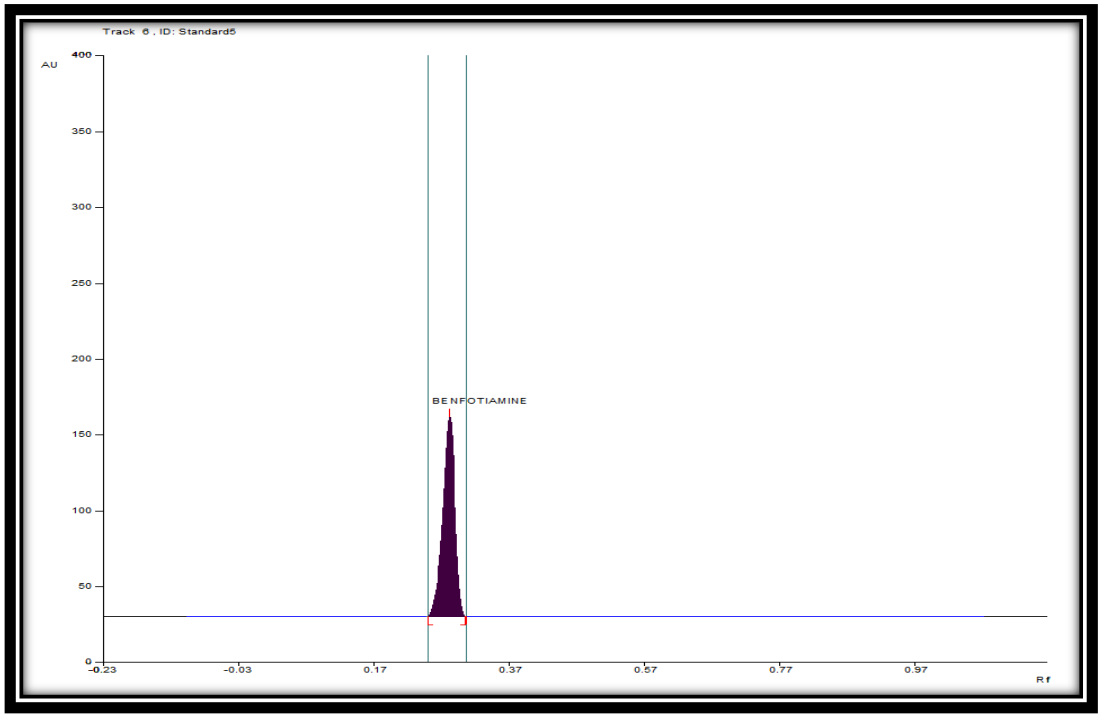
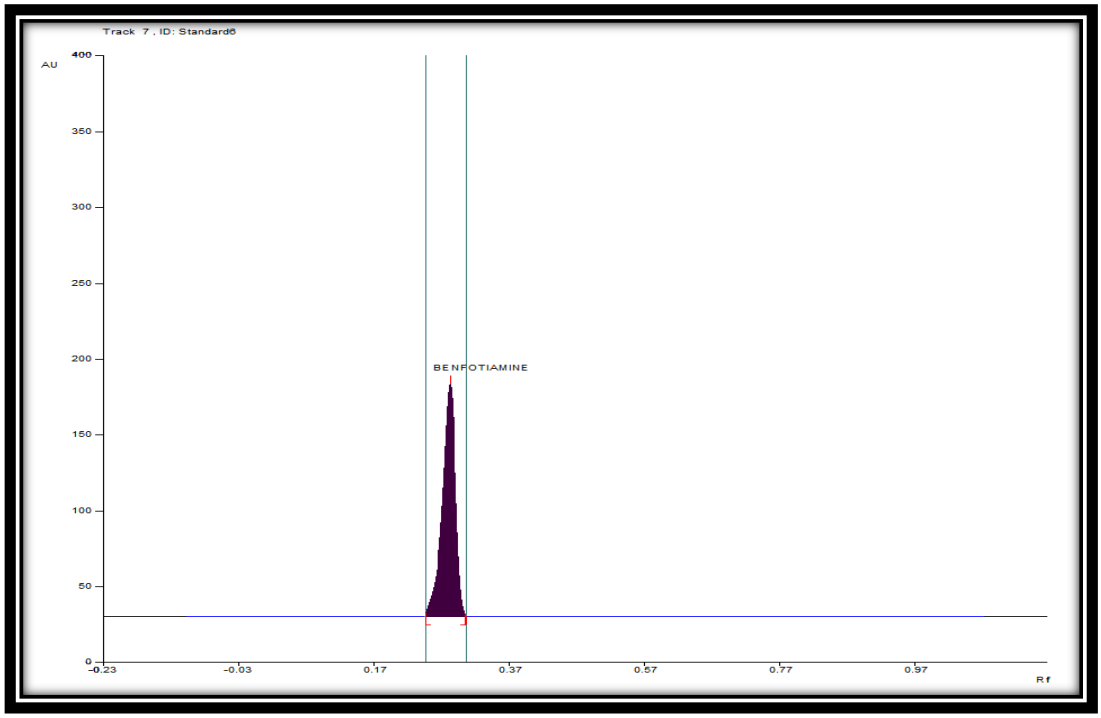


Fig 12: Benfotiamine 1200ng/spot



Precision

The results of repeatability of sample application and sample measurement are given in the table 10 and 11. The tables 12 and 13 summarizes the results of intra-day and inter-day precision. The % RSD values were found to be less than 2.

Table 10: Repeatability of sample application

Concentration(ng)	Peak area	%RSD
600	3658.4	0.57
	3666.3	
	3480.7	
	3618.8	
	3654.8	
	3643.4	

Table 11: Repeatability of measurement

Concentration(ng)	Peak area	%RSD
200	1628.2	1.13
	1640.2	
	1643.1	
	1682.3	
	1640.3	
	1652.9	

Table 12: Intraday precision

Concentration(ng)	Peak area	%RSD
400	2712.0	0.58
	2733.0	
	2701.5	
800	4562.3	0.36
	4571.7	
	4594.6	

Table 13: Interday precision

Concentration(ng)	Peak area	%RSD
400	2601.0	0.38
	2621.9	
	2617.0	
800	4503.6	0.61
	4559.1	
	4529.3	

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ were determined by applying decreasing amount of the drug in triplicate on the plate. The lowest concentration at which the peak is detected is called 'limit of detection' which was found out to be 10ng/spot.

The lowest concentration at which peak is quantified is called 'limit of quantification' which was found to be 50ng/spot.

Robustness

The robustness of the method is its ability to remain unaffected by small changes in the parameter such as ratio of mobile phase and saturation time. The method is said to be robust as minor variation (as shown below) not affected quantification.

Parameters	Observation
Ratio of mobile phase (Glacial Acetic acid: Methanol: Triethylamine(8:2v/v:2drops ±0.1ml,)	Very slight changes in R_f but no change in peak area
Saturation time(15 ±0.1, minutes)	peak symmetry retained.

Stability studies

Stability of chromatographic plate

When the developed method chromatographic plate was expose to atmosphere, the analytes are likely to decompose. Hence it is necessary to conduct stability studies of the plate.

Stability of the analyte on the plate was studied at different time intervals and peak areas were compared with peak area of freshly scanned plate. The developed plate was found to be stable for less than 24hrs, (stable 14).

Table 14: Stability of chromatographic plate

Time (hr)	Concentration(ng/spot)	Peak area
0	200	1628.2
	600	3658.4
12	200	1478.1
	600	3447.4
24	200	1425.1
	600	3406.6

ANALYSIS OF FORMULATION

Preparation of sample solution for benfotiamine:

Each of 20 tablets containing 100mg of Benfotiamine was taken for the study and average weight was determined. Quantity equivalent to 10mg Benfotiamine was weighed and transferred into a 10ml standard flask and after dissolving it was made up to volume with methanol. It was filtered and used for analysis.

Recording chromatogram:

The fixed chromatographic condition, a suitable volume of sample solution was applied on the precoated TLC plate. The plate was analyzed and chromatogram as recorded. The amount present per tablet, % label claim were calculated and shown in table 15, and chromatogram shown in fig 13.

Fig 13: Chromatogram of Benfotiamine formulation

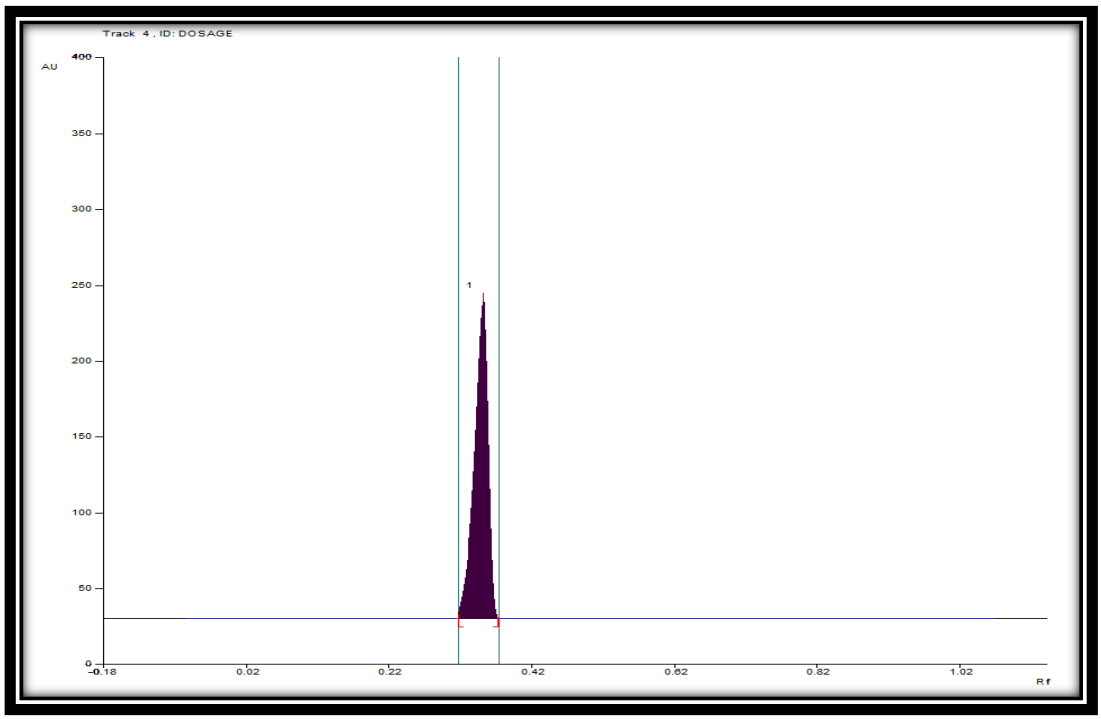


Table 15: Results of analysis formulation

Brand name	Amount of drug/tab		% label claim	% RSD	SEM
	Labeled	Found			
Benalgis	100mg	98.54mg	98.54%	0.98	0.13

***Average of six observations**

Rovers:

Recovery studies were carried out at 50% and 100% levels. The percentage recovery and percentage % RSD of the results were calculated and shown in table 16. The value proves the accuracy of the method

Table 16: recovery

Level	% recovery	% RSD	SEM
50%	92.36%	0.28	0.23
100%	95.25%	0.86	0.11

***Average of six observations**

Forced degradation studies:

HPTLC studies of samples obtained by stress testing of benfotiamine under different conditions suggested the following degradation behavior.

Acid hydrolysis:

Under given conditions of acid hydrolysis(0.01N HCL), it was found that at 0 hour 6.17% of the drug degraded (fig, 14) and on further degradation, i.e. after refluxing at 40ºC 2 hour 10% degradation was found out (fig, 15) and after highest 5 hour 13% drug degradation was found out (fig, 16). No degradant peak was found in the chromatogram.

Fig 14: Acid hydrolysis at 0 hour

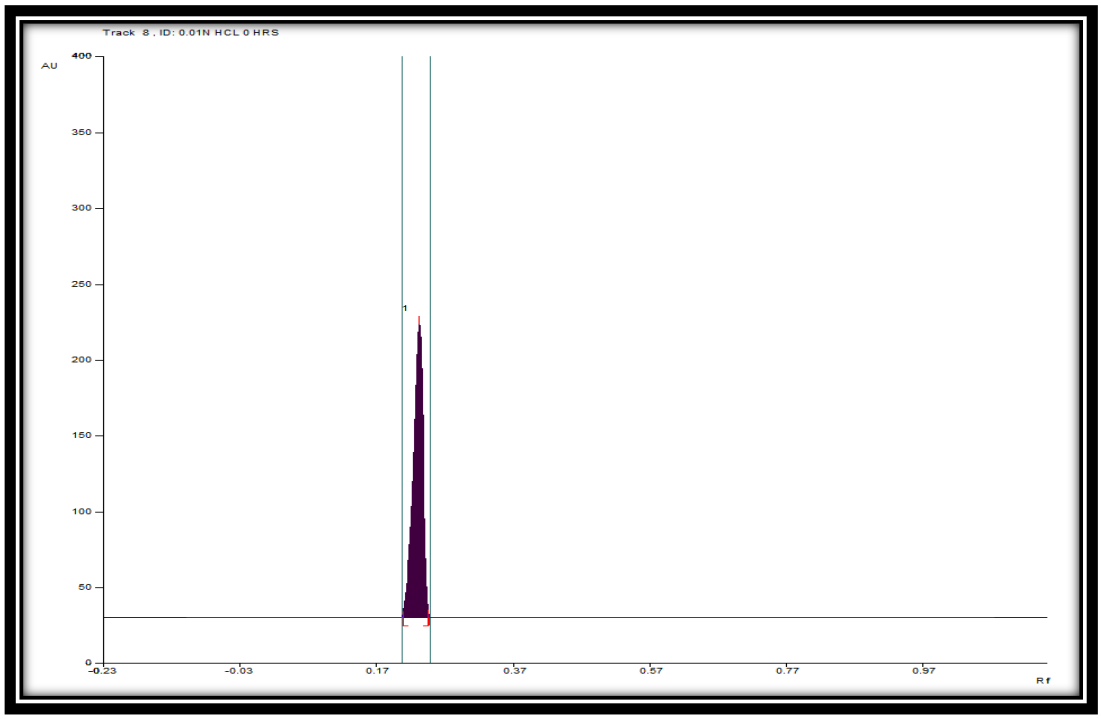


Fig 15: Acid hydrolysis at 2 hour

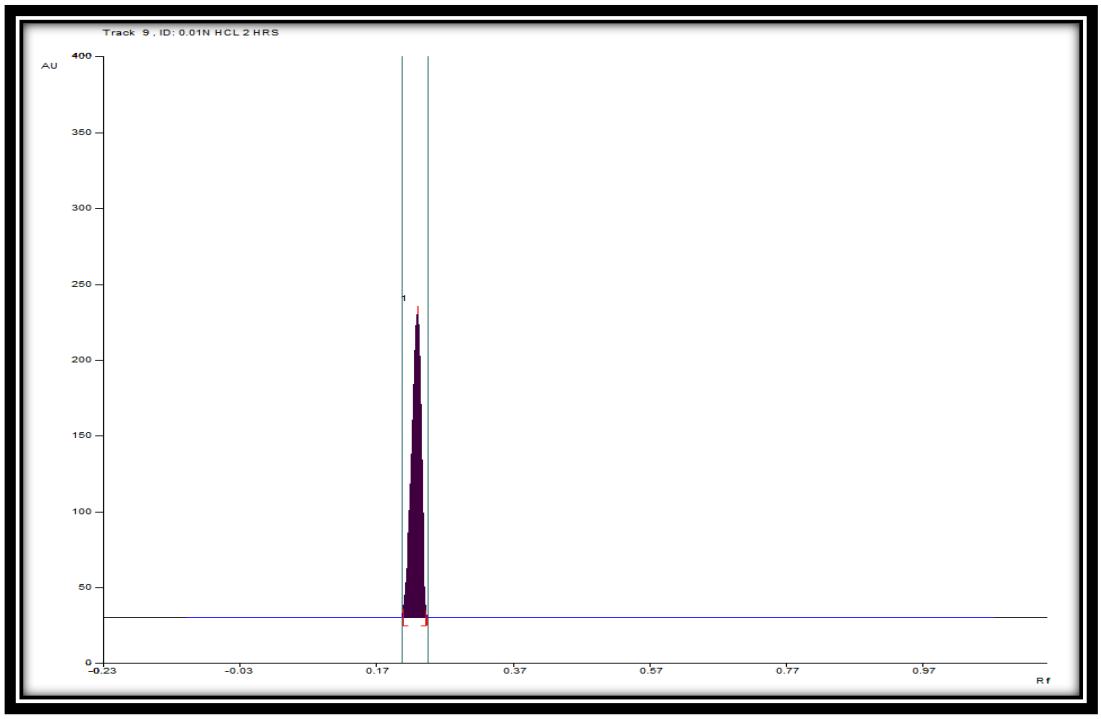
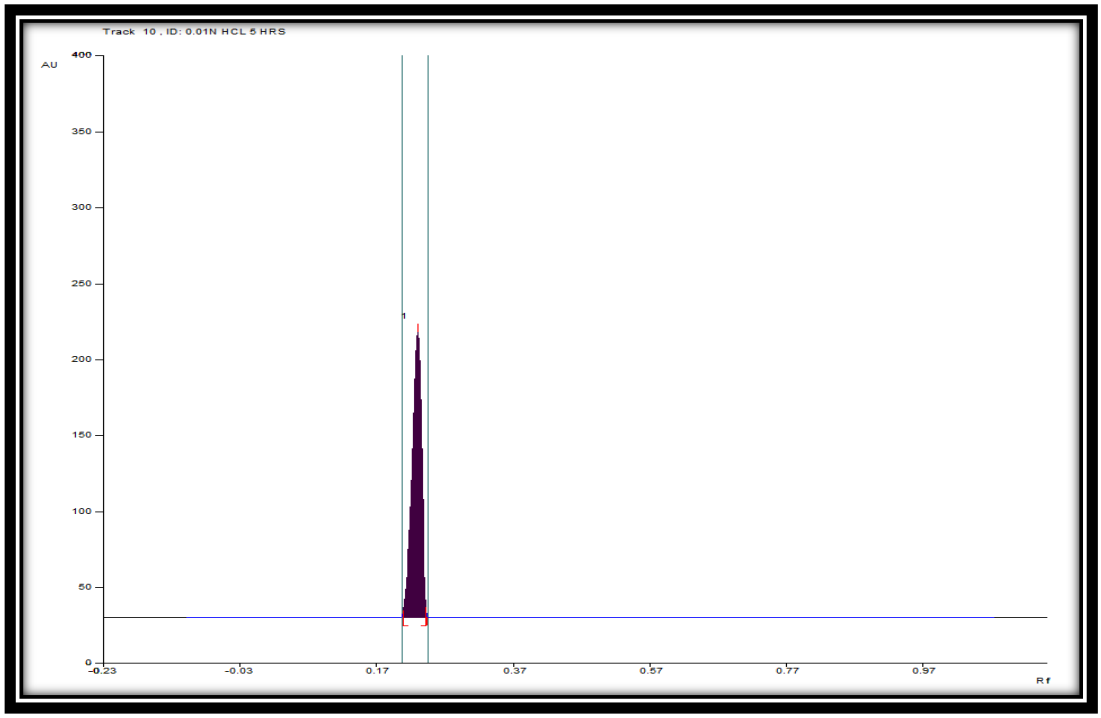


Fig 16: Acid hydrolysis at 5 hour



Alkaline hydrolysis:

In alkaline hydrolysis (0.01N NAOH), it was observed that 17.03% of the drug degraded at 0 hour, (fig, 17). On further degradation it was found that after refluxing at 40ºC for 5 hour 13.15% of the degraded (fig, 18). No degradation products were observed.

Fig 17: Alkaline hydrolysis at 0 hour

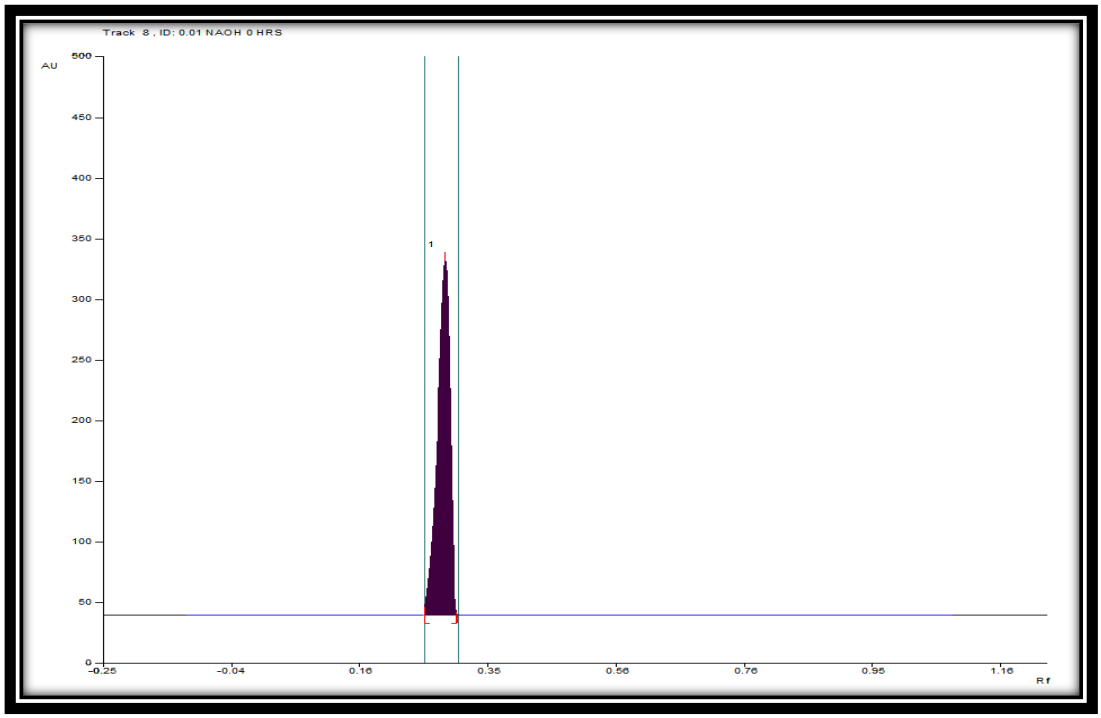
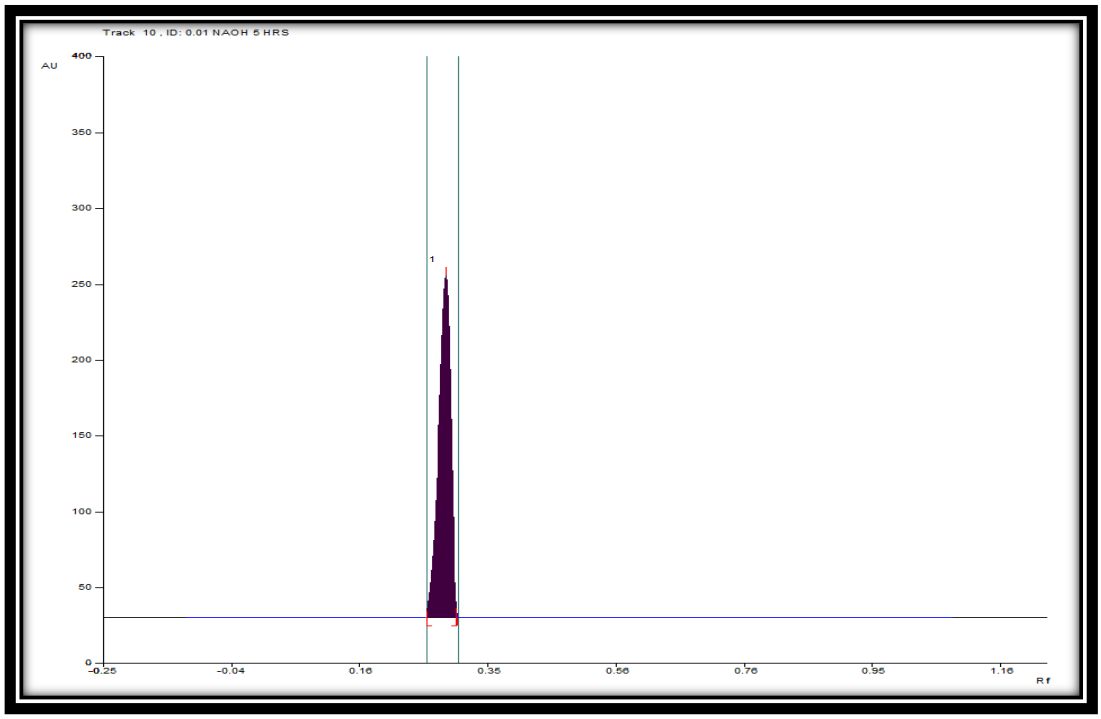


Fig 18: Alkaline hydrolysis at 5 hour



Oxidative degradation

The drug was also found to be susceptible to oxidation degradation. Here with 30% H_2O_2, at 0 hour it shows 25.16% degradation (fig 19), and up on further degradation, i.e., after refluxing for 5 hours at 40ºC it shows about 42.37% degradation without any degradation peak fig 20**.**

Fig 19: Oxidative degradation at 0 hour

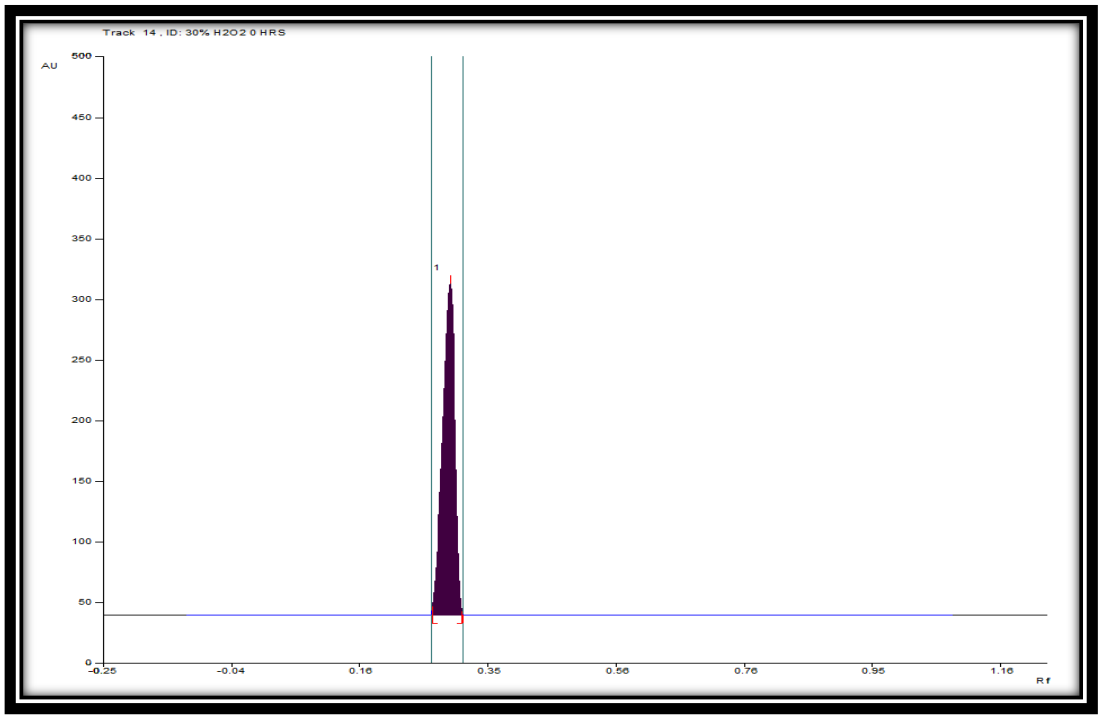
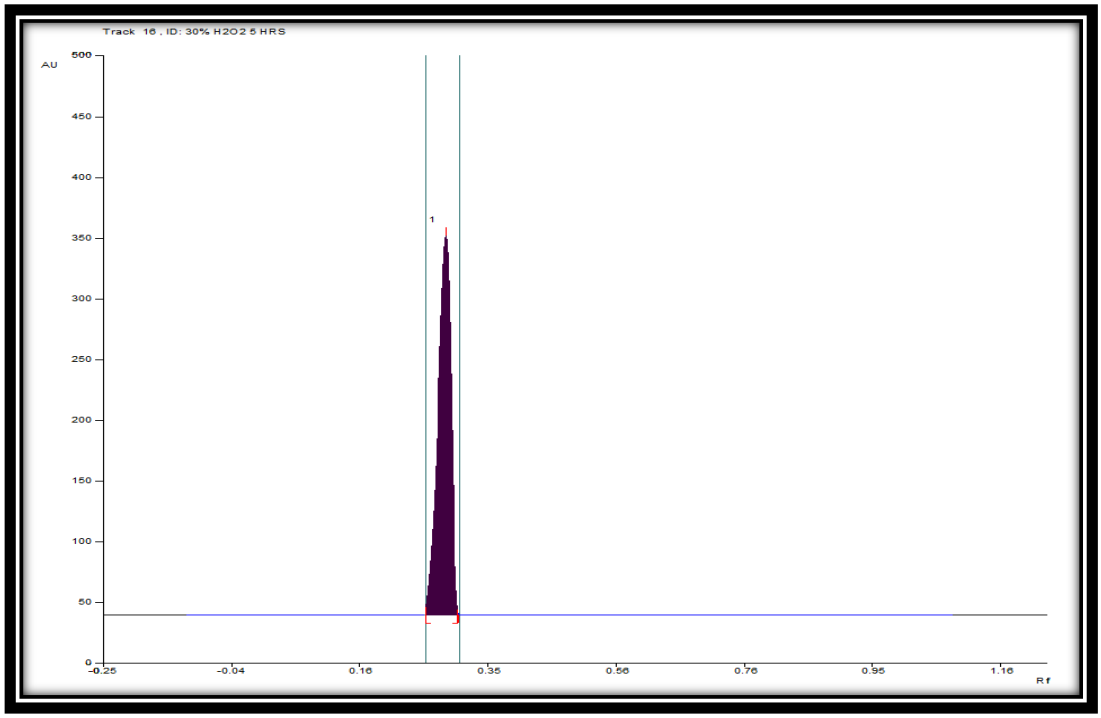


Fig 20: Oxidative degradation at 5 hour



The drug shows extensive degradation in acid, alkaline and oxidative degradation respectively (Table 17) presents the result of the studies.

Table 17: Results of degradation studies of Benfotiamine

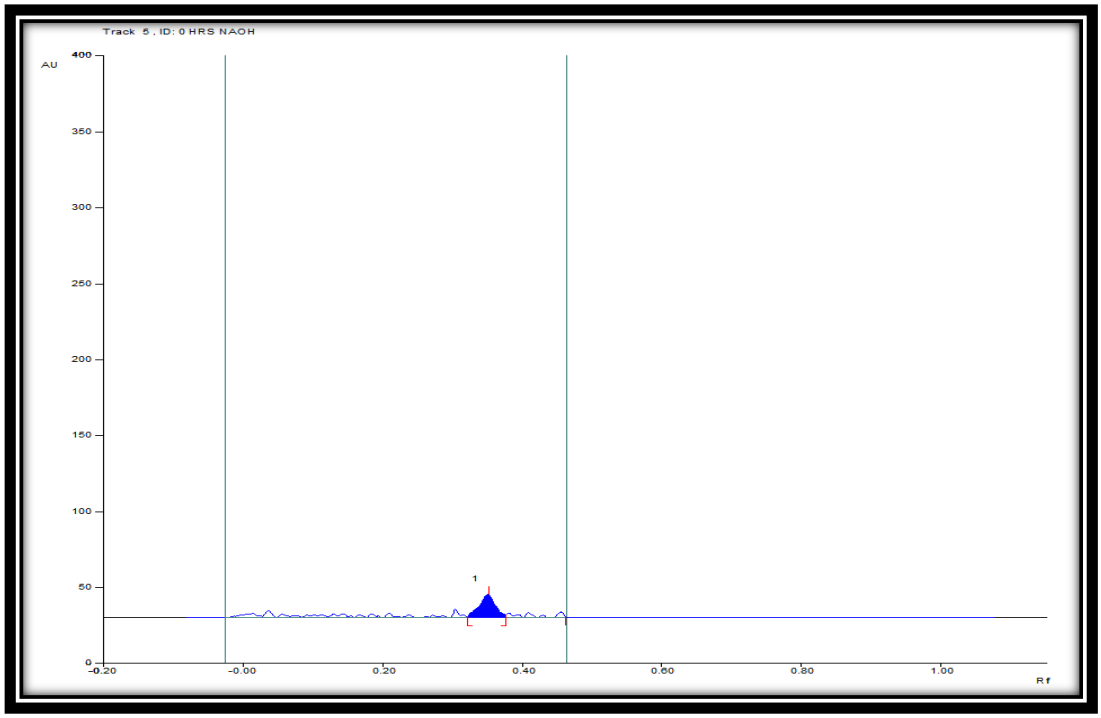
Type of stress	Condition	% degradation		R_f
		0 hour	5 hour	
Acid hydrolysis	0.01 N HCL	6.01%	11.30%	0.29
Alkaline hydrolysis	0.01 N NAOH	17.03%	5.44%	0.29
Oxidation	30% H_2O_2	25.16%	42.37%	0.29

Forced degradation studies resulted in considerable degradation as evidenced by the reduction in the peak area when subjected to acid/base/oxidation degradation. The degradants formed were not detected but showed a marked decrease in the peak area of the parent drug. No significant change in the R_f value shows the evidence for the analyte peak after significant degradation.

Degradation of placebo in formulation:

The fixed chromatographic condition was applied to estimate placebo inactive ingredients. The placebo was subjected for acid/base hydrolysis and oxidation and used the some excipients in degradation study. The results chromatogram given below, fig 21. There was an interference seen at R_f 0.36 which was not interfering with benfotiamine R_f value (0.29).

Fig 21: Alkaline hydrolysis placebo at 0 hour



Degradation of Benfotiamine in formulation:

From the various stress samples prepared 0.6µl was spotted on TLC plate in triplicates analyzed using fixed chromatographic conditions described earlier. (Fig, 22,-24).

Fig 22: Acid hydrolysis of formulation

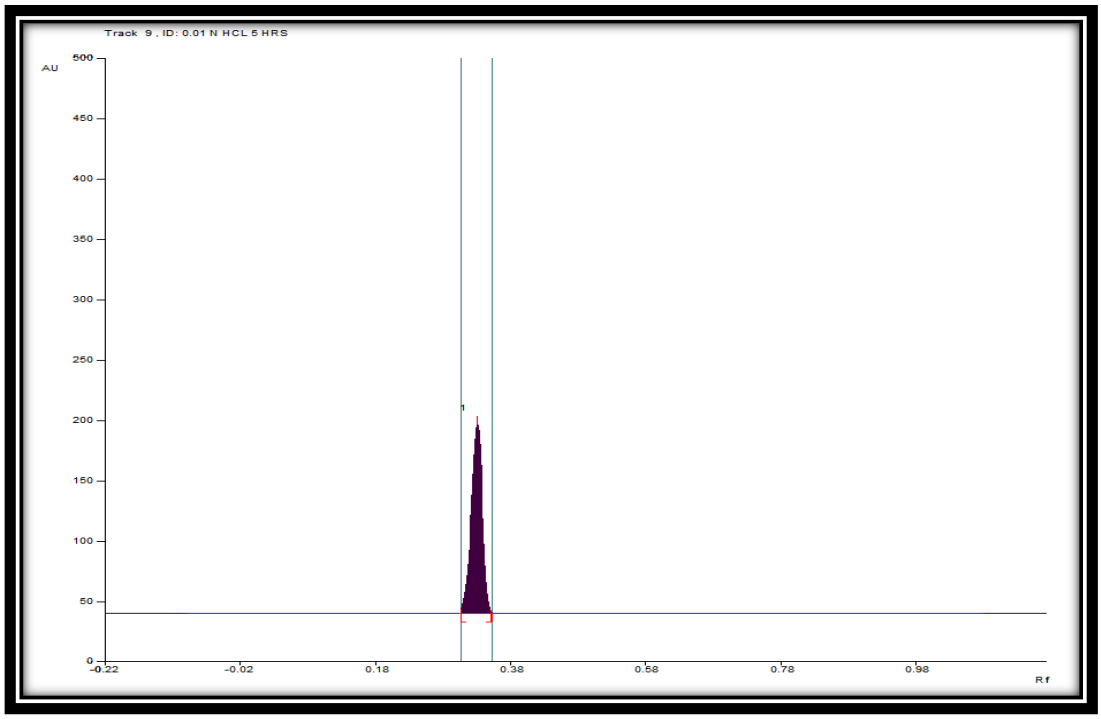


Fig 23: Alkaline hydrolysis of formulation

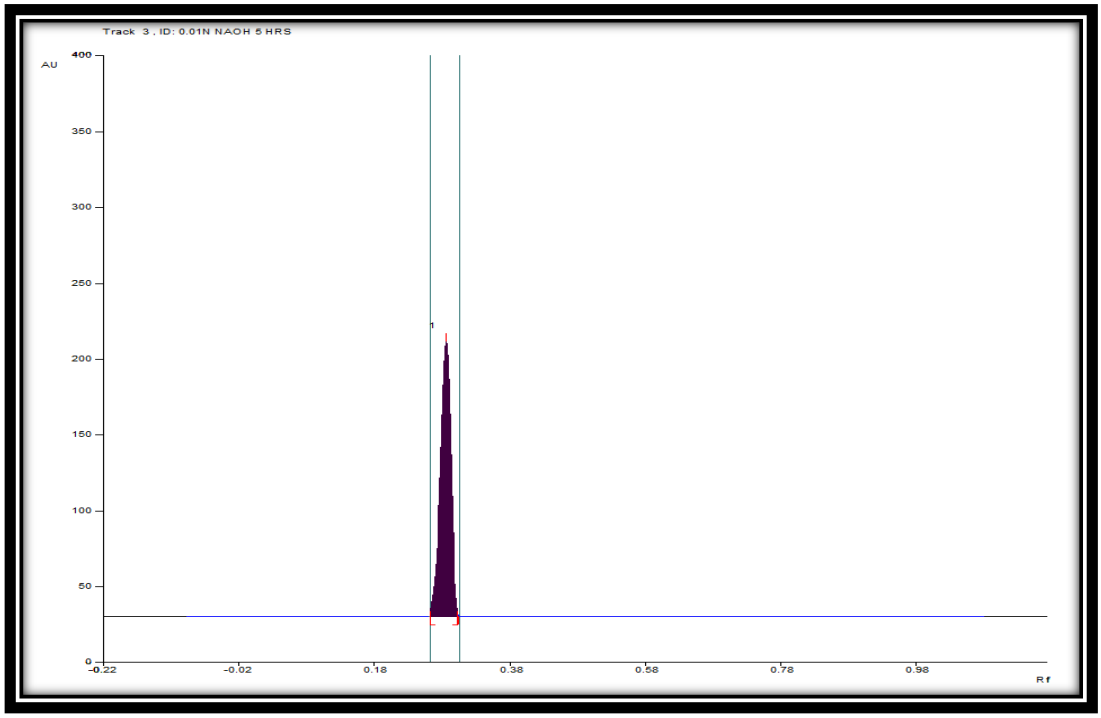
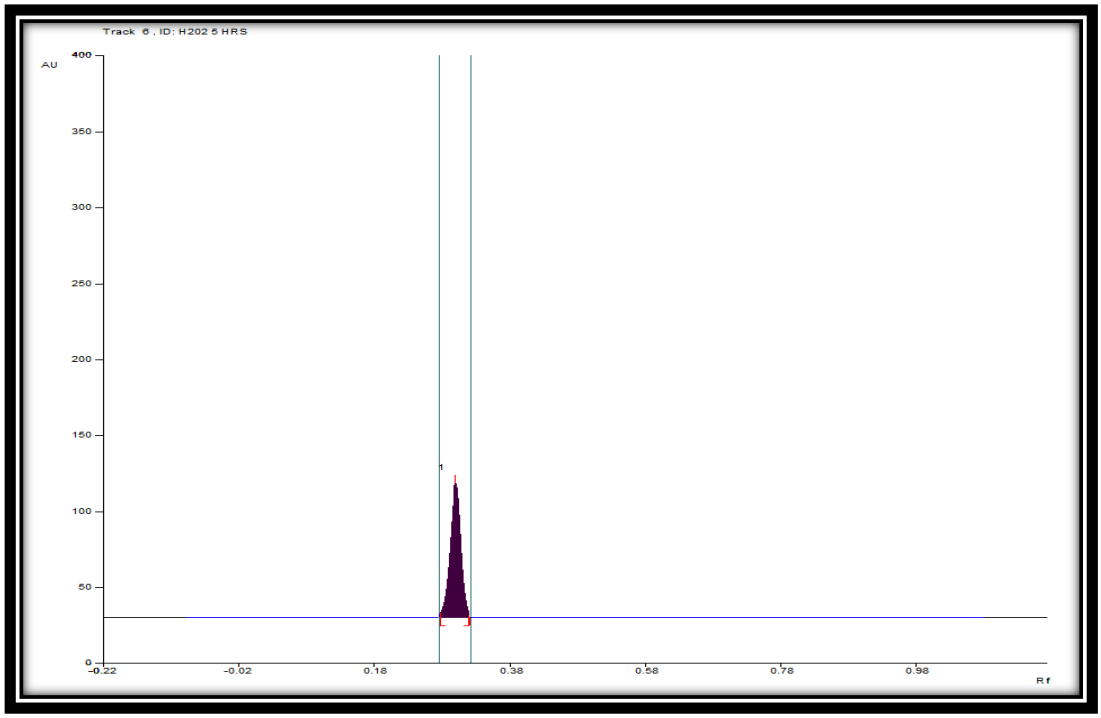


Fig 24: Oxidative degradation of formulation



Amount of benfotiamine reminded after degradation studies:

Degradation studies	Maximum degradation time		% of Benfotiamine remaining interferents
	Temperature	Retention time	
Acid hydrolysis	40ºC 4 hours	R_f -29	7.79
Alkaline hydrolysis	40ºC 4 hours	R_f -29	61.23
Oxidative degradation	40ºC 4 hours	R_f -29	66.25

III. DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR ESTIMATION OF BENFOTIAMINE IN COMBINATION WITH PYRIDOXAMINE DIHYDROCHLORIDE AND RESVERATROL IN COMBINED DOSAGE FORM

Among various mobile phase system tried the one contain a mixture of Glacial acetic acid: Ether: Acetone: Triethylamine: Tetra butyl ammonium bromide (5.5:1.5:3 % v/v: 5 drops: 2 drops) gave compact dense spot was chosen for the estimation of benfotiamine by HPTLC method. The plate used was pre-coated silica gel $G_{60}F_{254}$. The method was for the validated and details given below.

Linearity and range:

A 200µg/ml and 100µg/ml of BEN, PYR and RES was prepared in methanol. Aliquots of this solution (1-6µl) were applied on the plate. The plate was developed, scanned and peak areas were noted. Linear regression data showed good correlation coefficient over a concentration range of (200-1200ng/spot and 100-600ng/spot). The calibration graphs are shown in fig 25, 26 and 27. The slope, intercept, and correlation coefficient values were in noted respectively (table 18). The spectra recorded on HPTLC scanner and the standard densitograms are shown in fig.28 to 31, and calibration data were shown table, 19, 20, and 21.

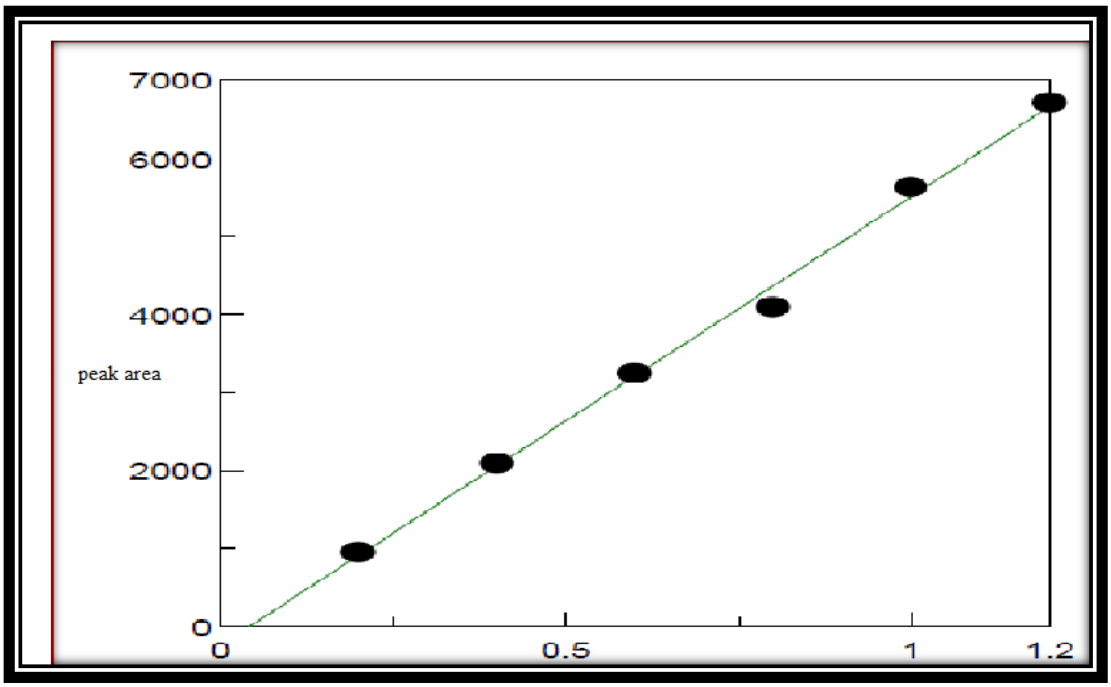
Table 18: Regression data

Linear regression	Benfotiamine	Pyridoxamine	Resveratrol
Slope	5753	3053.43	11440.3
Intercept	-241.4	-90.4	-696.2
Correlation coefficient	0.998	0.997	0.998

Table 19: Calibration data for Benfotiamine

Concentration (ng/spot)	Peak area
200	948.5
400	2093.5
600	3242.6
800	4089.8
1000	5626.1
1200	6713.7

Fig 25: Calibration graph of Benfotiamine (200-1200ng/spot)



Concentration (ng/ml)

Table 20: Calibration data for Pyridoxamine dihydrochloride

Concentration (ng/spot)	Peak area
100	281.8
200	680.5
300	902.3
400	1330.9
500	1691.4
600	1607.4

Fig 26: Calibration graph of Pyridoxamine dihydrochloride (100-600ng/spot)

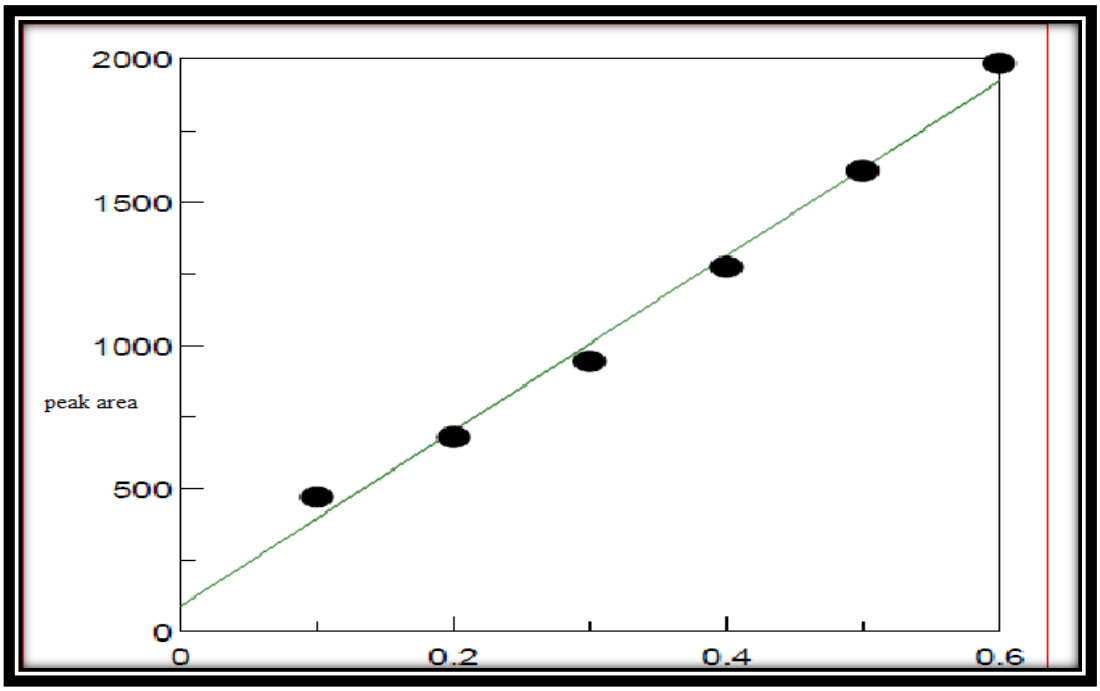


Table 21: Calibration data for Resveratrol

Concentration(ng/spot)	Peak area
100	454.8
200	1441.9
300	3310.1
400	3923.9
500	4977.8
600	6138.7

Fig 27: Calibration graph of Resveratrol (100-600ng/spot)

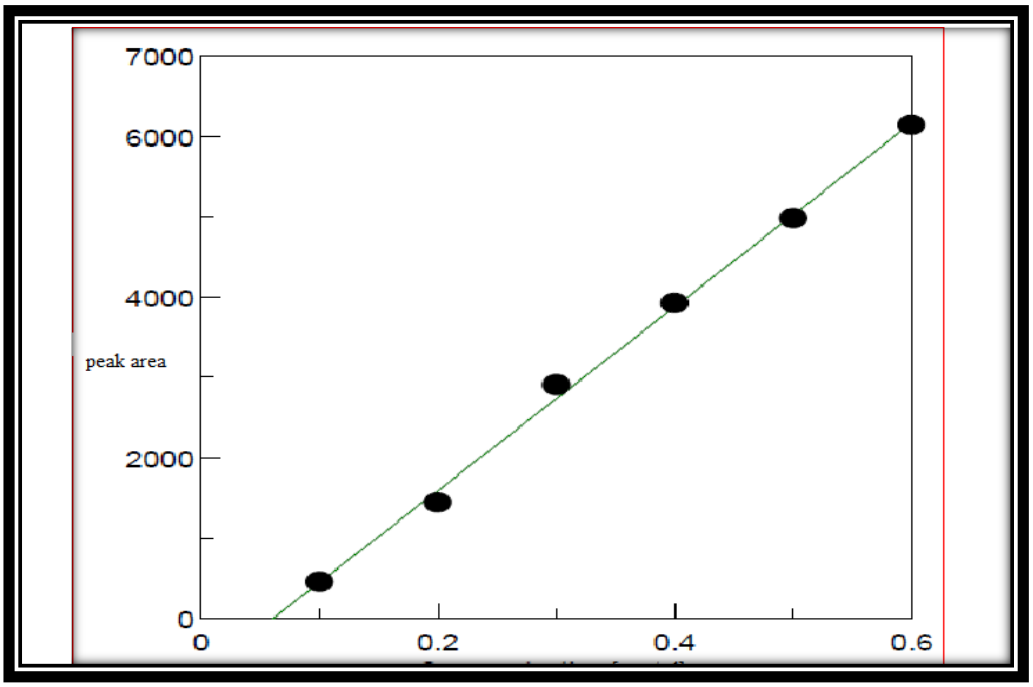


Fig 28: Spectrum of Benfotiamine

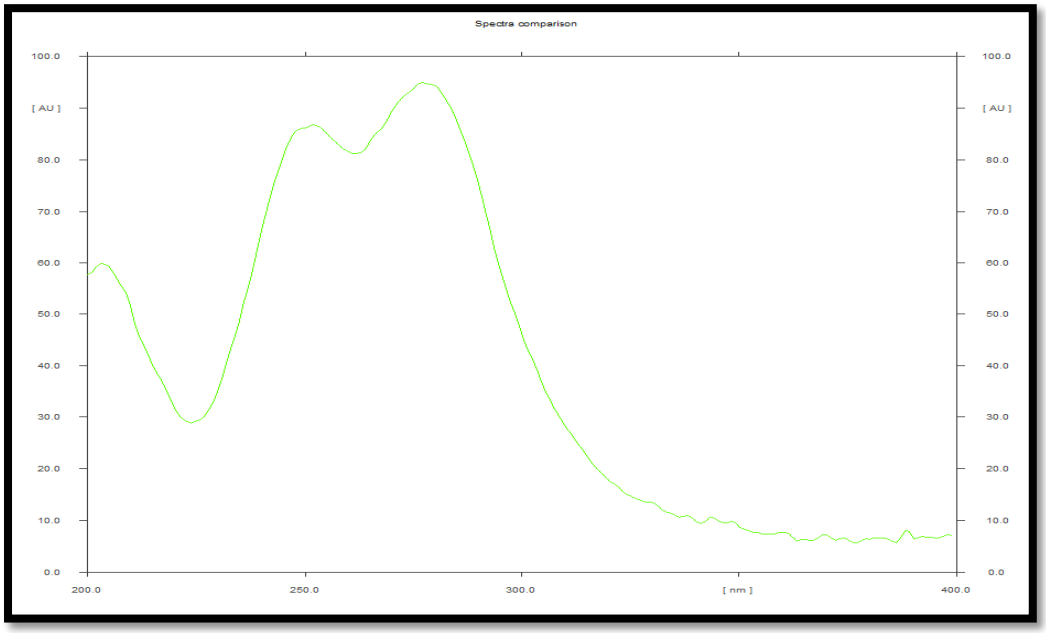


Fig29: Spectrum of Pyridoxamine dihydrochloride

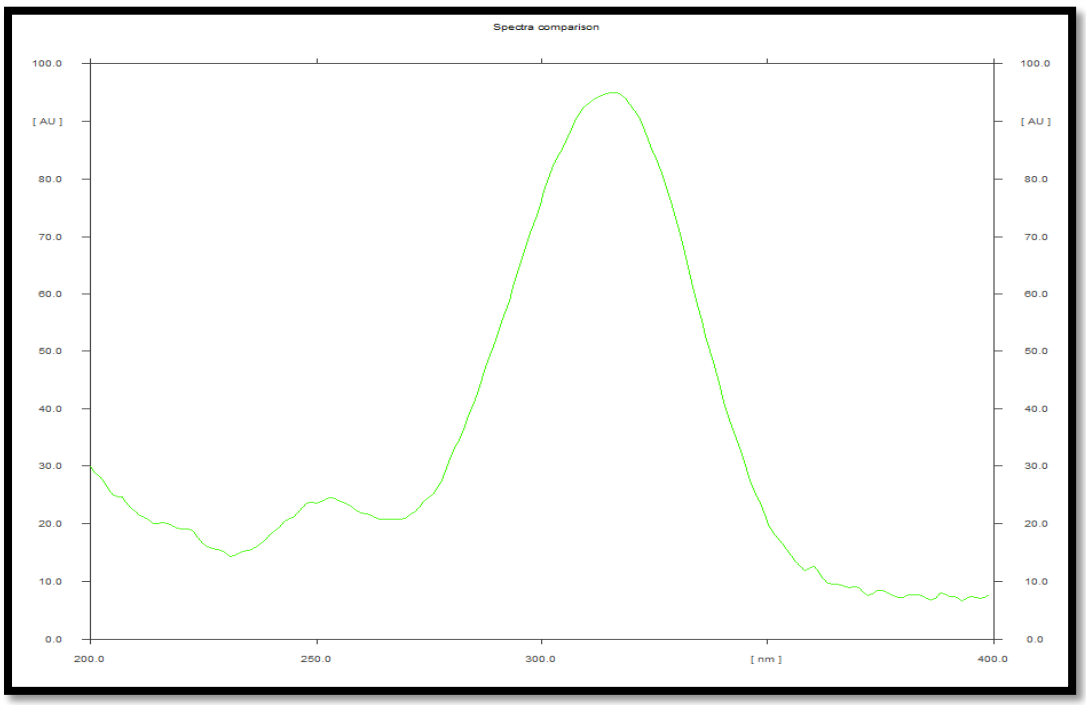


Fig 30: Spectrum of resveratrol

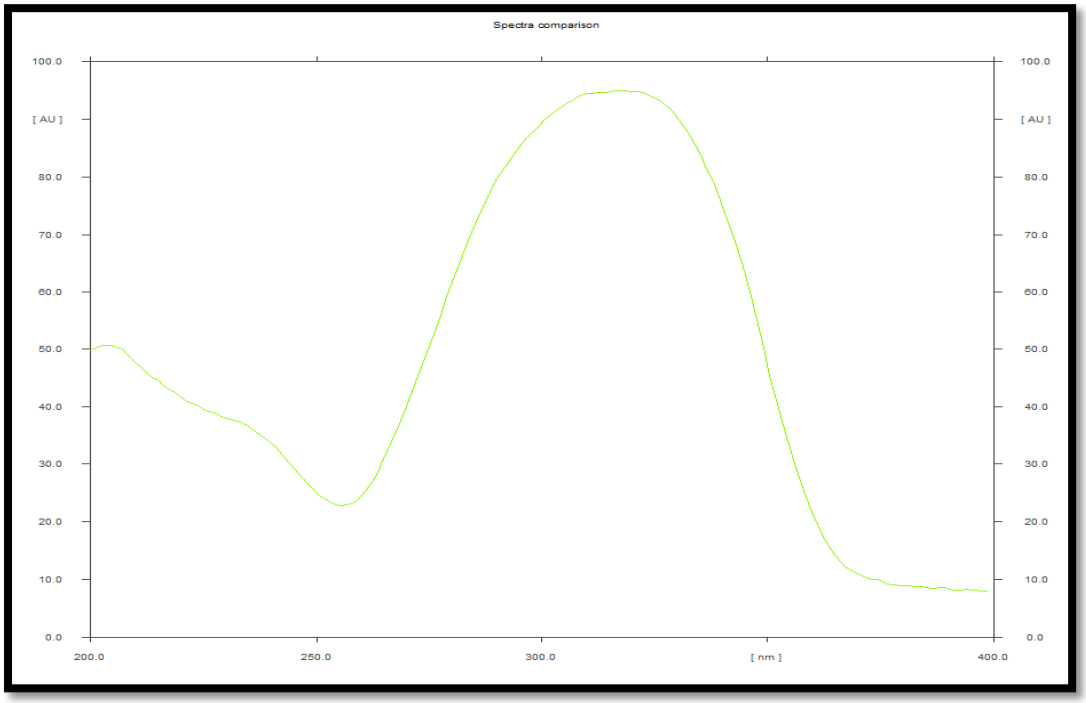
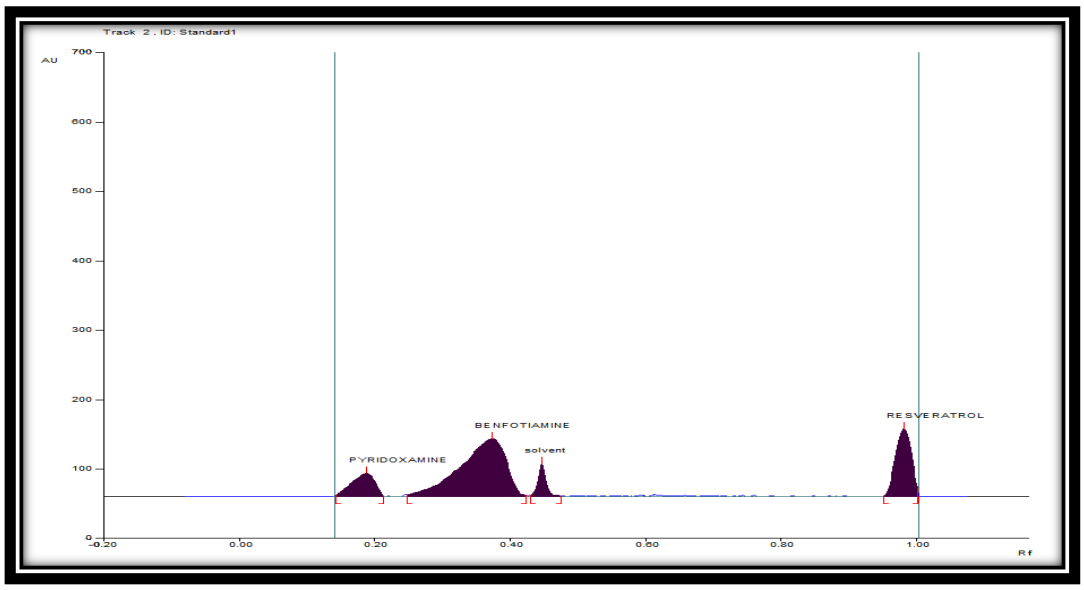


Fig 31: Chromatogram of Benfotiamine, Pyridoxamine dihydrochloride and Resveratrol



Precision

The method precision was obtained by determining the assay by preparing six samples of selected concentration (600 & 1000ng/spot) of Benfotiamine and 300 & 500ng/spot Pyridoxamine dihydrochloride and Resveratrol. The % RSD calculated for method precision of inter-day, intra-day (table 22, 23 and 24) and repeatability (repeatability of sample measurement and sample application respectively) are shown in table 25, 26 and 27.

Table 22: Intra-day and inter-day precision of Benfotiamine

Concentration (ng/spot)	Peak area		%RSD	
	Intra-day	Inter-day	Intra-day	Inter-day
600	3242.6	3230.8	0.58	1.34
	3218.7	3291.3		
	3256.5	3263.3		
1000	5626.1	5523.7	0.19	0.16
	5617.4	5545.7		
	5639.2	5587.4		

Table 23: Intra-day and inter-day precision of Pyridoxamine dihydrochloride

Concentration (ng/spot)	Peak area		%RSD	
	Intra-day	Inter-day	Intra-day	Inter-day
300	719.8	704.7	0.62	0.62
	714.5	708.3		
	710.9	699.5		
500	1258.7	1153.8	0.64	1.44
	1262.2	1132.2		
	1264.8	1121.5		

Table 24: Intra-day and inter-day precision of Resveratrol

Concentration (ng/spot)	Peak area		% RSD	
	Intra-day	Inter-day	Intra-day	Inter-day
300	1048.7	964.4	1.44	0.94
	1062.3	953.6		
	1079.3	946.5		
500	2189.3	2256.8	1.94	1.32
	2110.5	2280.8		
	2126.1	2220.9		

Table 25: Repeatability of sample measurement and sample application of Benfotiamine

Concentration (ng/spot)	Peak area		% RSD	
	Sample measurement	Sample application	Sample measurement	Sample application
400	1531.5	1413.5	0.74	1.36
	1525.1	1452.0		
	1499.3	1416.6		
	1516.6	1459.6		
	1519.8	1429.2		
	1509.4	1449.4		

Table 26: Repeatability of sample measurement & sample application of Pyridoxamine dihydrochloride

Concentration (ng/spot)	Peak area		%RSD	
	Sample measurement	Sample application	Sample measurement	Sample application
200	602.1	584.2	0.74	0.84
	614.5	599.0		
	604.7	594.9		
	608.0	593.2		
	602.8	589.4		
	607.3	592.7		

Table 27: Repeatability of sample measurement & sample application of Resveratrol

Concentration (ng/spot)	Peak area		%RSD	
	Sample measurement	Sample application	Sample measurement	Sample application
200	1235.4	1355.0	1.18	0.73
	1254.2	1345.4		
	1224.6	1342.6		
	1214.0	1332.1		
	1247.3	1336.2		
	1237.2	1327.6		

Robustness

The robustness of the method is its ability to remain unaffected by small changes in the parameter such as ratio of mobile phase and saturation time. The method is said to be robust as minor variation (as shown below) not affected quantification.

Parameters	Observation
Ratio of mobile phase ± 0.1 (Acetic acid: Ether: Acetone: Triethylamine: tetra butyl ammonium bromide(5:5:1.5:3:5 drops: 2drop ± 0.1ml)	Slight changes in R_f, but no different in peak area
Saturation time10 (±0.1,minutes,)	Peak symmetry retained.

Stability studies

Stability of chromatographic plate

When the developed method chromatographic plate was exposed to atmosphere, the analytes are likely to decomposed. Hence it was necessary to conduct stability studies of the plate.

Stability of the analyte on the plate was studied at different time intervals and peak areas were compared with peak area of freshly scanned plate. The developed plate was found to be stable for less than 24hrs, table 28, 29, and 30.

Table 28: Benfotiamine

Time (hr)	Concentration(ng/spot)	Peak area
0	400 800	2093.5 4089.8
6	400 800	1616.6 3248.0
22	400 800	1452.0 3134.2

Table 29: Pyridoxamine dihydrochloride

Time (hr)	Concentration(ng/spot)	Peak area
0	200 400	680.8 1330.9
6	200 400	374.9 925.6
22	200 400	359.4 825.6

Table 30: Resveratrol

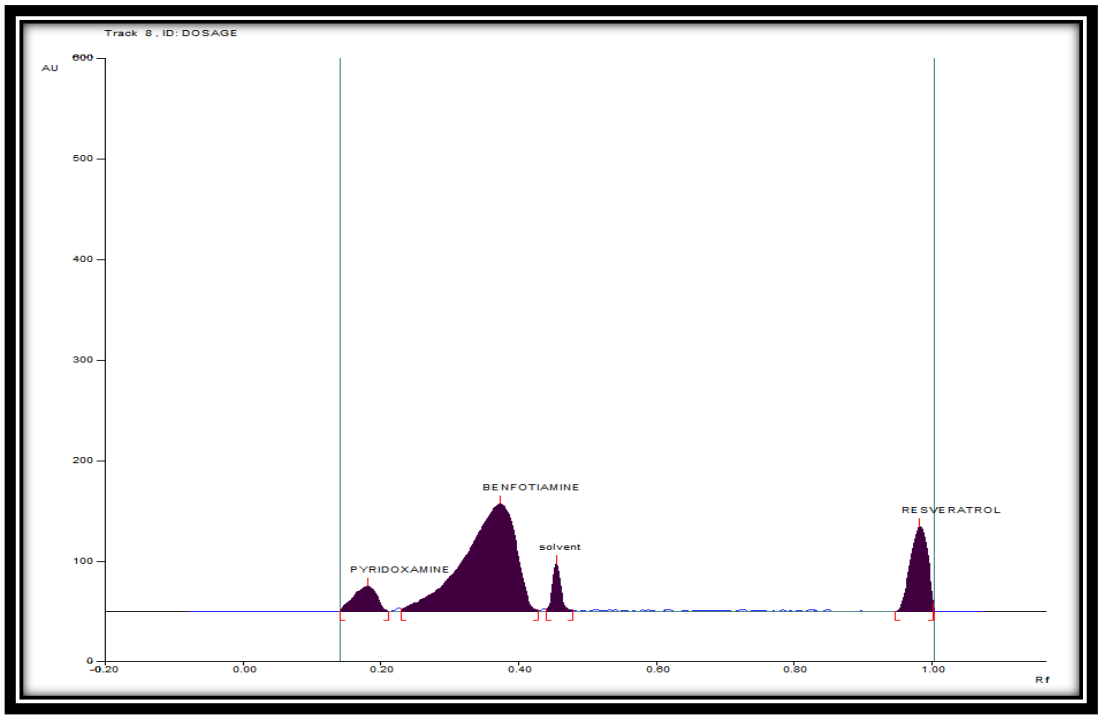
Time (hr)	Concentration(ng/spot)	Peak area
0	200 400	1441.9 3923.9
6	200 400	942.7 3278.8
22	200 400	912.5 3014.4

ANALYSIS OF FORMULATION

Preparation of sample solution for Benfotiamine, Pyridoxamine dihydrochloride, Resveratrol

20 tablets, each containing 50mg of Benfotiamine, 25mg of Pyridoxamine dihydrochloride and 25mg of Resveratrol were taken for the studies and average weight were determined. Quality equivalent to sample was weighed and transferred to 10ml volumetric flask extracted and made up to volume with methanol. It was filtered and used for analysis, (fig, 32).

Fig 32: Chromatogram of Benfotiamine, Pyridoxamine dihydrochloride and Resveratrol formulation



Recording chromatogram

With the fixed chromatographic condition, a suitable volume of sample solutions was applied on the precoated TLC plate. The plate was analyzed and chromatogram was recorded, fig 32.

Peak areas of sample chromatograms were noticed and the amount of drugs was calculated, table 31.

Table 31: Results analysis of formulation

Brand name	Amount of drug/tab		% label claim	% RSD	SEM
Ageless	Labeled	Found	96.42%	0.74	0.17
Benfotiamine	50mg	48.21mg			
Pyridoxamine dihydrochloride	25mg	24.15mg	96.60%	0.45	0.03
Resveratrol	25mg	23.48mg	93.92%	0.65	0.08

***average of six observations**

IV. DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF BENFOTIAMINE IN COMBINATION WITH PYRIDOXAMINE DIHYDRO-CHLORIDE AND RESVERATROL TABLET FORMULATION

The drugs is a polar in nature hence RP-HPLC method and C_{18} column was used for the HPLC method development for the estimation of benfotiamine in combination of pyridoxamine dihydrochloride and resveratrol.

Selection of wavelength:

Good analytical separation can be obtained only by careful selection of wavelength for the detection (fig.33, 34 and 35). The smooth spectrum obtained from which 254nm was selected for the drug. The overlay spectrum of all three drugs shown in fig 36.

Fig 33: Spectrum of Benfotiamine

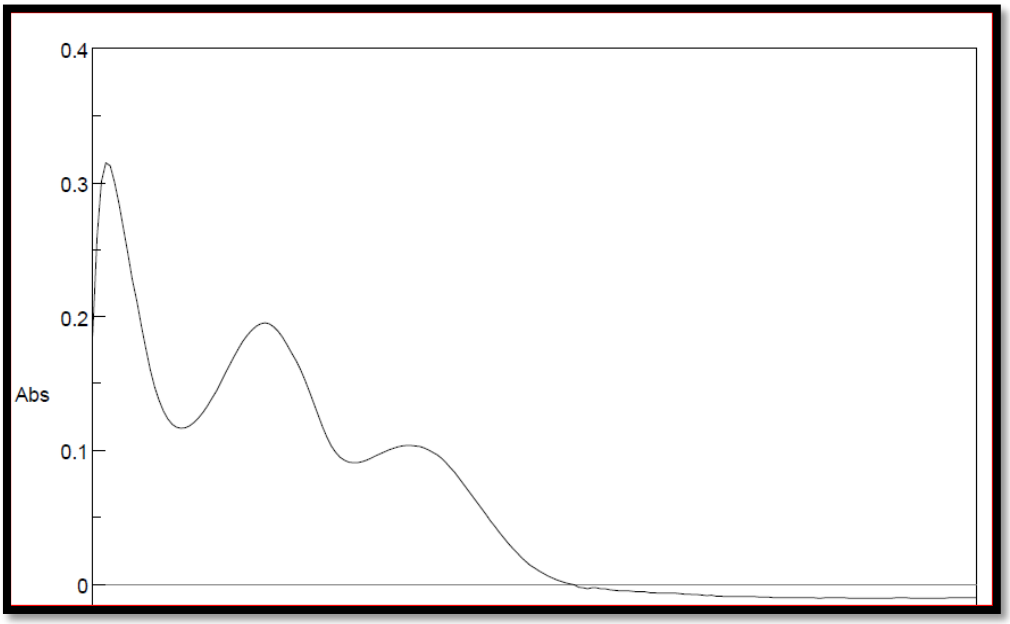


Fig 34: Spectrum of Pyridoxamine dihydrochloride

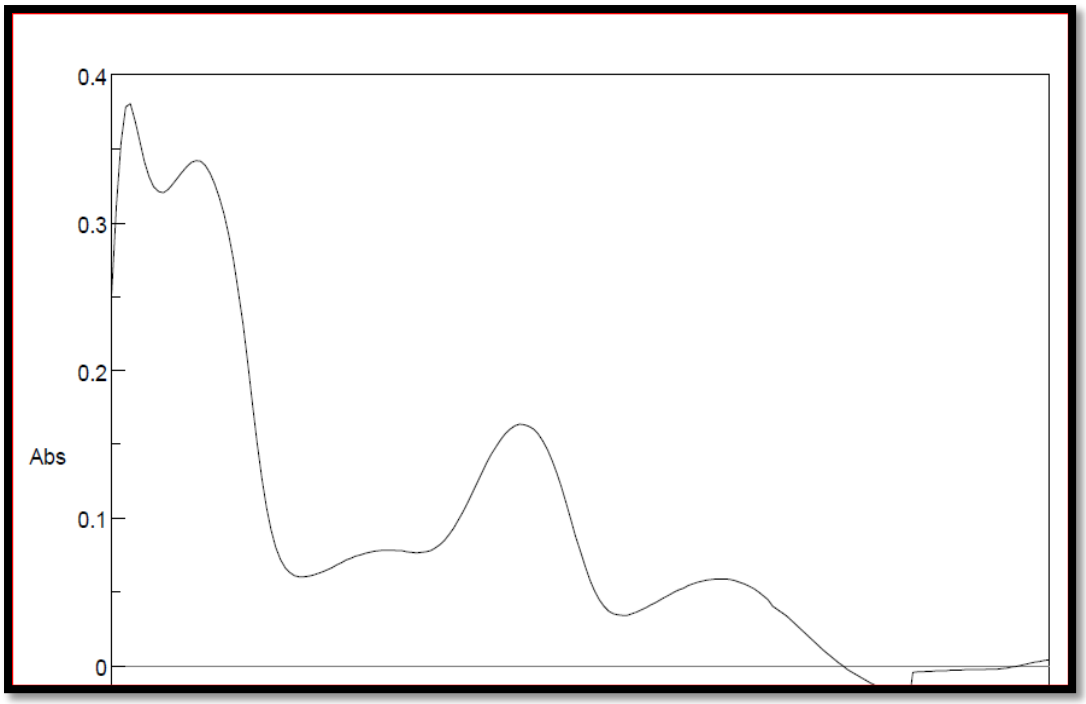


Fig 35: Spectrum of Resveratrol

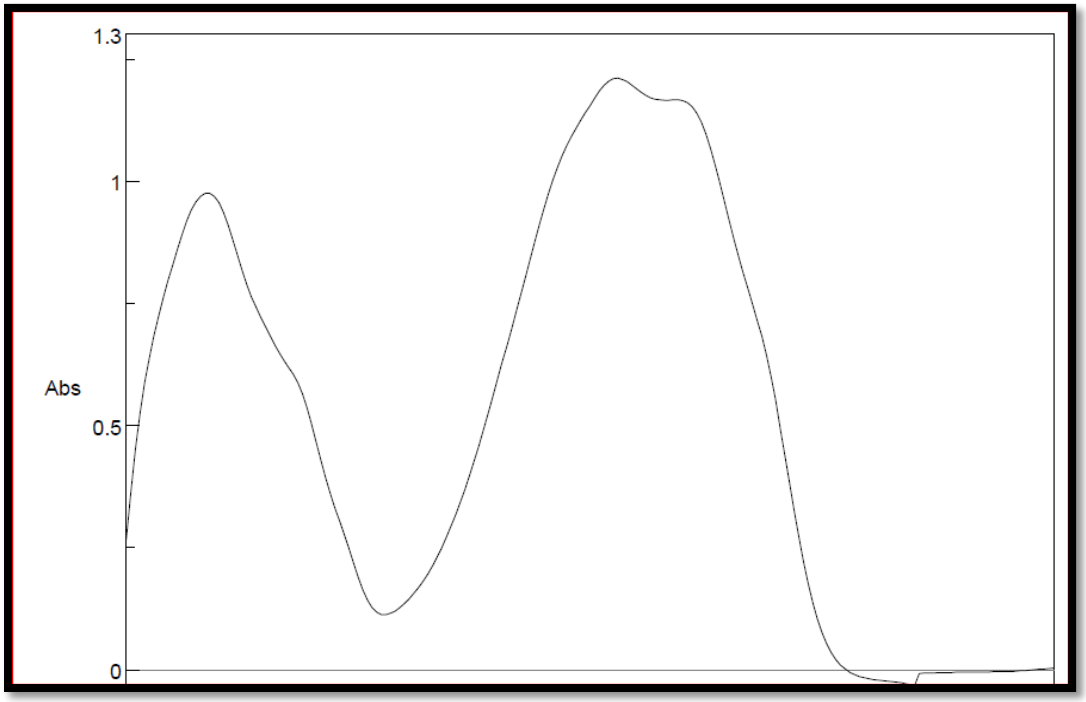
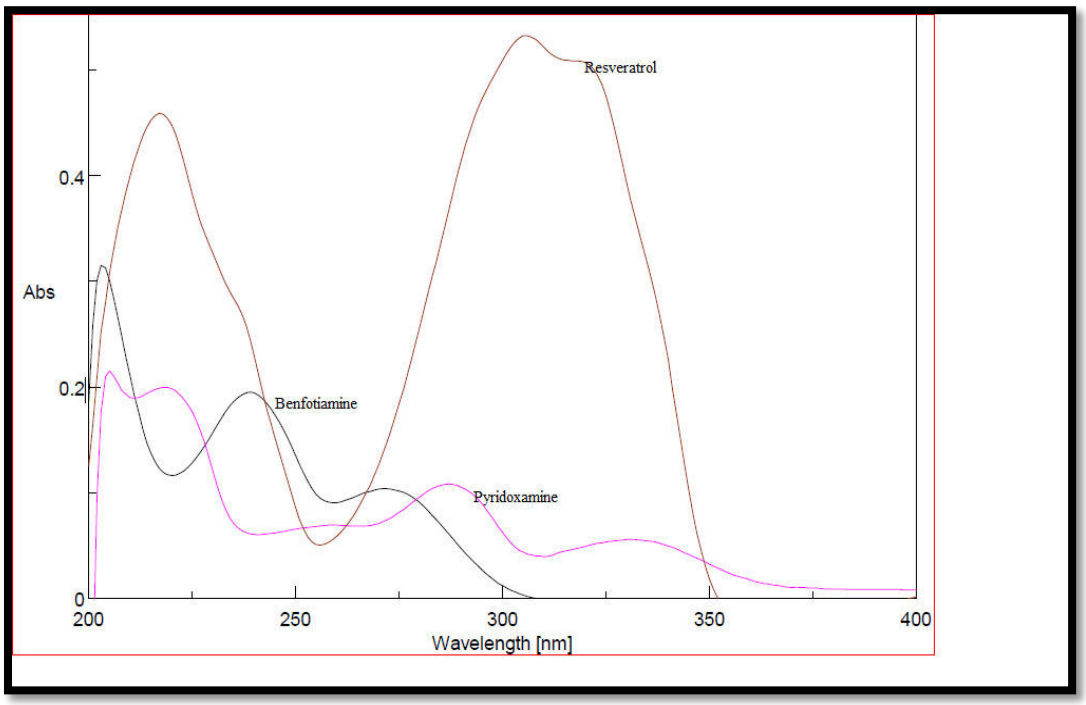


Fig 36: Overlay spectrum of Benfotiamine, Pyridoxamine dihydrochloride & Resveratrol



Selection of mobile phase:

For developing HPLC method, mobile phase system containing potassium dihydrogen phosphate (pH adjusted with ortho phosphoric acid): methanol and acetonitrile was tried at different ratios and different pH. The observations are given below, table 32.

Table 32: Selection of mobile phase

Mobile phase	Ratio % v/v	Effect of pH	Observation
10mM ammonium acetate: Acetonitrile	10:90	4.65	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is same BEN- symmetry peak PYR- symmetry peak RES- peak tailing
10mM ammonium acetate: Acetonitrile	20:80	4.62	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is same BEN- symmetry peak PYR- peak tailing RES- peak tailing
10mM ammonium acetate: Acetonitrile	20:80	3.05	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is same EN- peak tailing PYR- peak tailing RES- peak tailing
10mM ammonium acetate: Acetonitrile	20:80	3.50	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is same BEN- peak tailing PYR- peak tailing RES- peak tailing

Mobile phase	Ratio % v/v	Effect of pH	Observation
10mM ammonium acetate: Acetonitrile	20:80	2.80	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is same BEN- peak tailing PYR- peak tailing RES- peak tailing
10mM ammonium acetate: Acetonitrile	40:60	2.80	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is same BEN- peak tailing PYR- peak tailing RES- peak tailing
10mM potassium dihydrogen phosphate: Methanol	20:80	5.20	Benfotiamine, pyridoxamine dihydrochloride & resveratrol is resolution less than 2 BEN- peak tailing PYR- peak tailing RES- peak tailing
10mM potassium dihydrogen phosphate: Methanol	20:80	4.50	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is same BEN- peak tailing PYR- peak tailing RES- peak tailing
10mM potassium dihydrogen phosphate: Methanol	30:70	4.50	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is same BEN- peak tailing PYR- peak tailing RES- peak tailing
10mM potassium dihydrogen phosphate:	50:25+ 25	5.21	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is separate

Mobile phase	Ratio % v/v	Effect of pH	Observation
Methanol+ Acetonitrile			BEN- peak spilt PYR- peak tailing RES- peak symmetry
10mM potassium dihydrogen phosphate: Methanol+ Acetonitrile	50:35+ 15	5.22	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is separate BEN- peak spilt PYR- peak tailing RES- peak symmetry
10mM potassium dihydrogen phosphate: Methanol+ Acetonitrile	60:20+ 20	5.21	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is separate BEN- peak spilt PYR- peak symmetry RES- peak symmetry
10mM potassium dihydrogen phosphate: Methanol+ Acetonitrile	65:17. 5+17.5	5.21	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is separate BEN- peak tailing PYR- peak symmetry RES- peak symmetry
10mM potassium dihydrogen phosphate: Methanol+ Acetonitrile	70:15+ 15	5.24	Benfotiamine, pyridoxamine dihydrochloride & resveratrol retention time is separate BEN- peak symmetry PYR- peak fronting RES- peak symmetry
10mM potassium dihydrogen phosphate: Methanol+ Acetonitrile	**70:15 +15**	**4.90**	**Good resolution between all drugs with acceptable peak shape.**

Linearity and range:

A calibration graph was plotted with measured peak areas against concentration. From the graph it was found that BEN, PYR and RES shows good linearity in the concentration between 10-50µg/ml for BEN, 5-25µg/ml for PYR and RES. The peak area of these solutions was measured at 254nm. The slope, intercept, and correlation coefficient values were calculated respectively (table 33). The linearity table is shown in table 34, 35 and 36. The linear graph and standard chromatogram obtained are shown in fig 37 to 44.

Table 33: Regression data

Linear regression	Benfotiamine	Pyridoxamine dihydrochloride	Resveratrol
Slope	51090.8	97602.1	12488.1
Intercept	-23973.5	-16901.5	-10335.9
Correlation coefficient	0.999	0.997	0.999

Table 34: Calibration data for Benfotiamine

Concentration (µg/ml)	Peak area
10	276467
20	596201
30	761997
40	1020219
50	1240952

Fig 37: Calibration graph of Benfotiamine (10-50µg/ml)

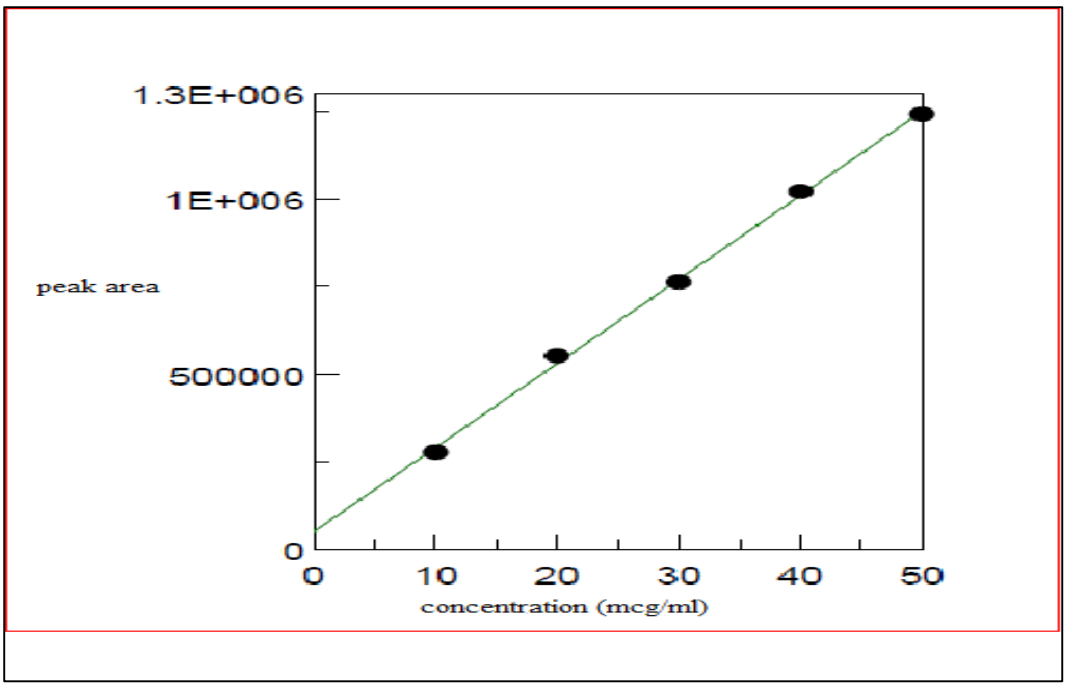


Table 35: Calibration data for Pyridoxamine dihydrochloride

Concentration (µg/ml)	Peak area
5	172106
10	273347
15	365260
20	427182
25	517725

Fig 38 : Calibration graph of Pyridoxamine dihydrochloride (5-25 µg/ml)

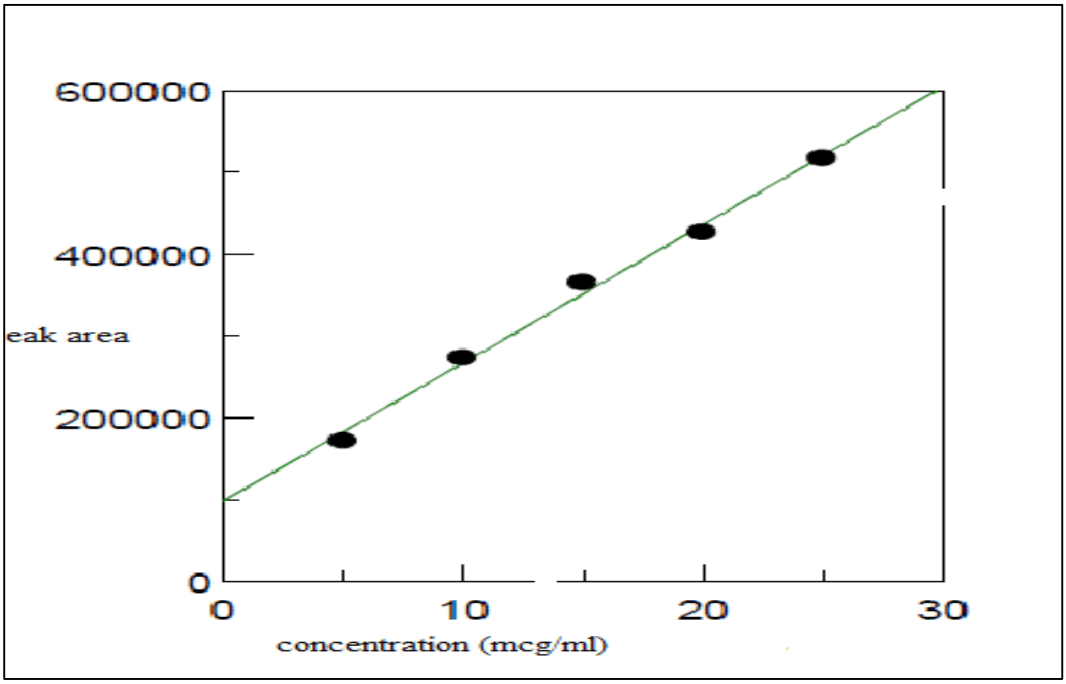


Table 36: Calibration data for Resveratrol

Concentration (µg/ml)	Peak area
5	75019
10	155132
15	200955
20	254657
25	323061

Fig 39: Calibration graph of Resveratrol (5-25µg/ml)

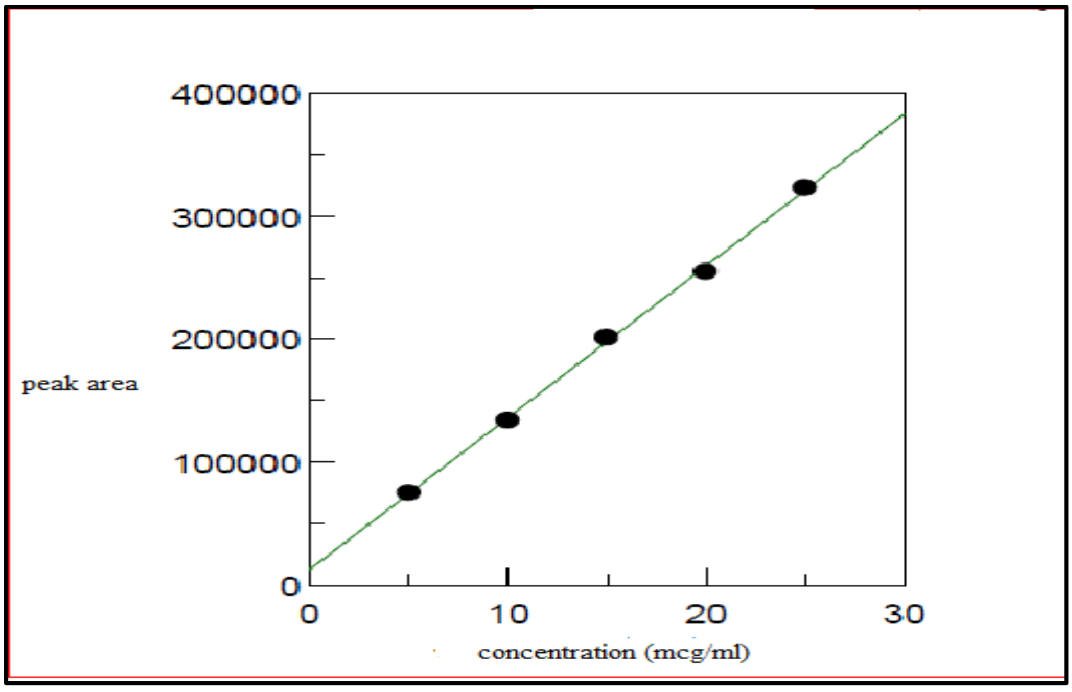


Fig 40: Chromatogram of BEN (10µg/ml), PYR and PYR (5µg/ml)

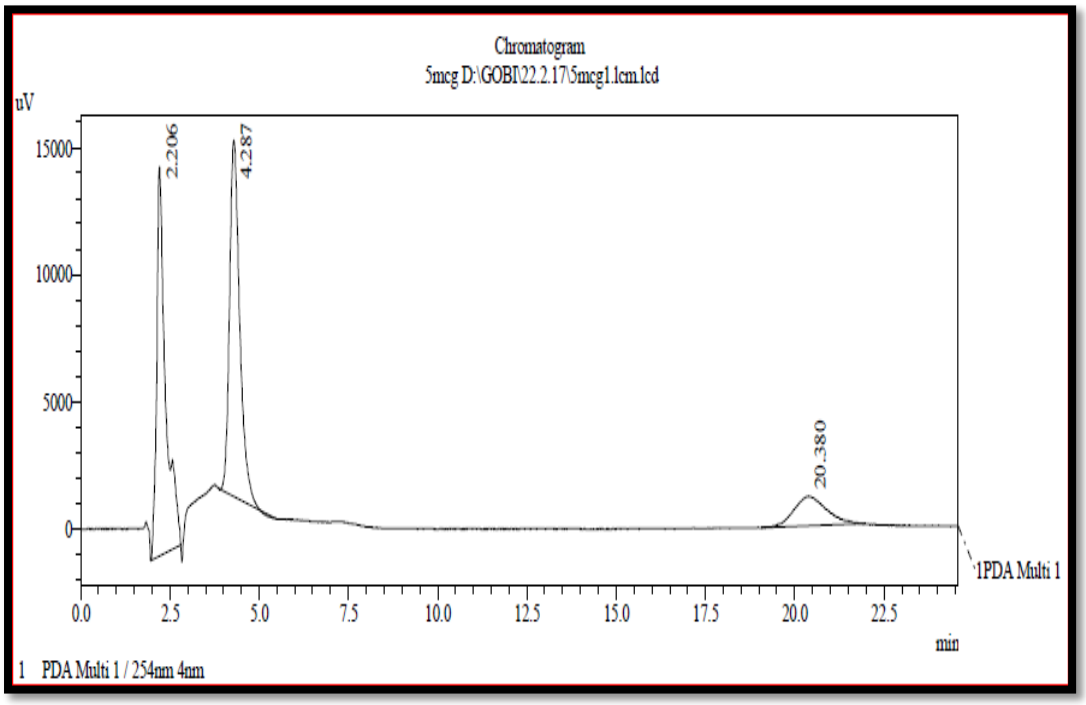


Fig 41: Chromatogram of BEN (20µg/ml), PYR and PYR (10µg/ml)

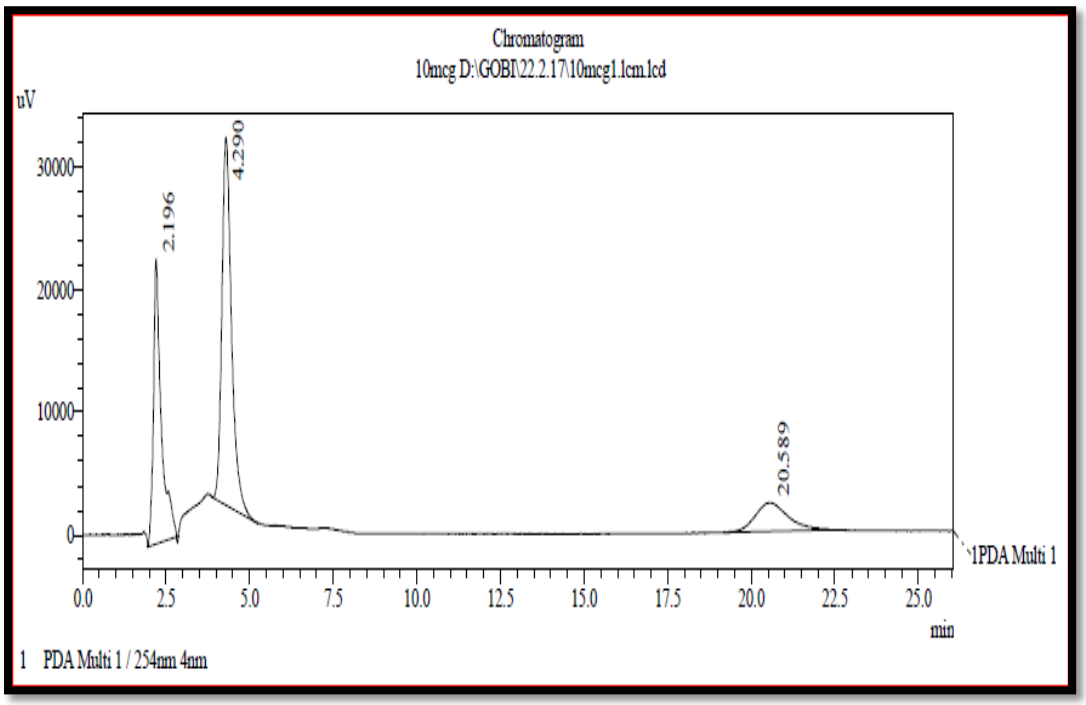


Fig 42: Chromatogram of BEN (30µg/ml), PYR and PYR (15µg/ml)

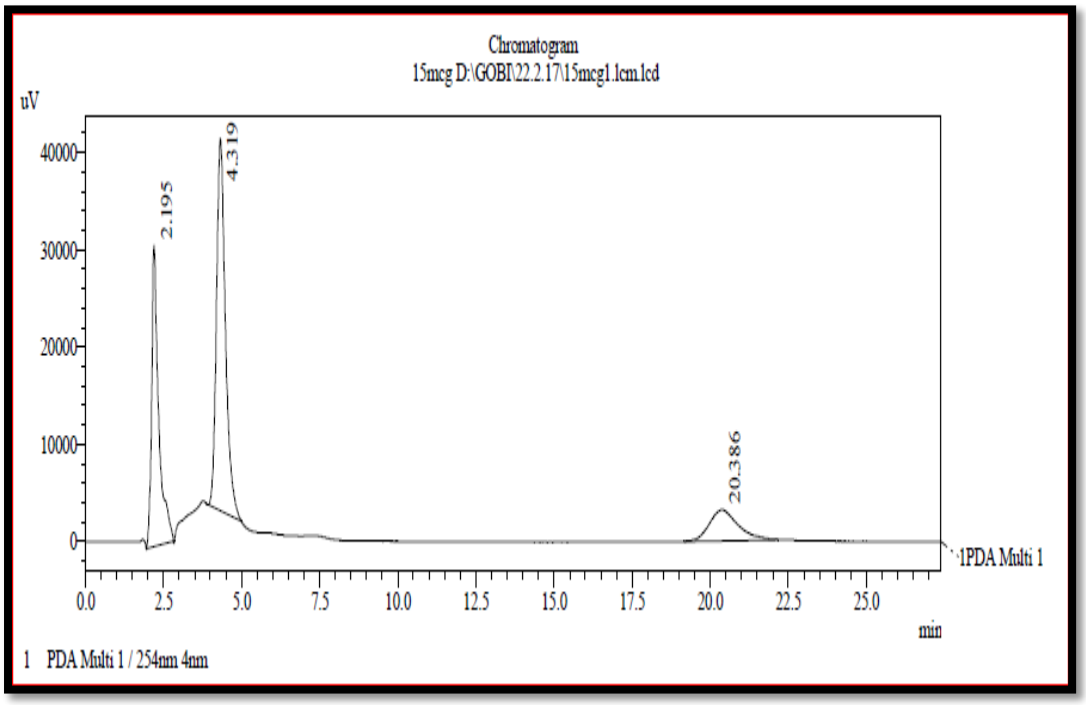


Fig 43: Chromatogram of BEN (40µg/ml), PYR and PYR (20µg/ml)

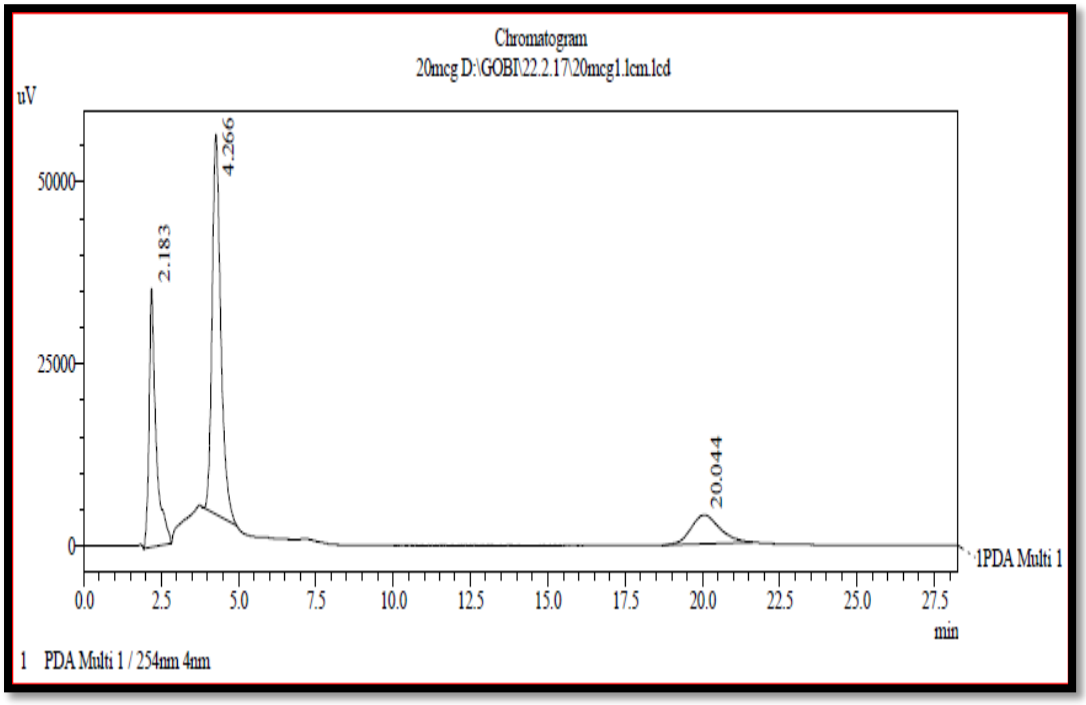
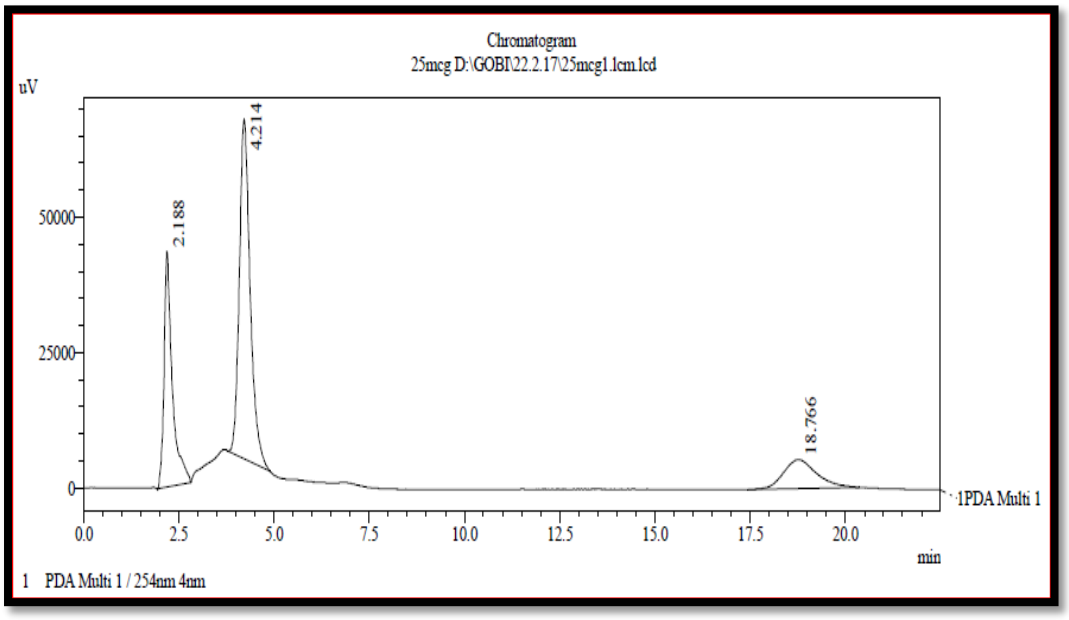


Fig 44: Chromatogram of BEN (50µg/ml), PYR and PYR (25µg/ml)



Precision:

Intra-day and inter-day precision:

Intra-day and inter-day precision was determined by injecting standard solutions in between linearity range (20 and 30µg/ml for BEN, 10 and 15µg/ml for PYR and RES) were injected for three times and % RSD was calculated (table 37, 38 and 39).

Table 37: Intraday and interday precision of Benfotiamine

Concentration (µg/ml)	Peak area		%RSD	
	Intraday	Interday	Intraday	Interday
20	593475	589287	0.16	0.06
	592474	589054		
	591473	588542		
30	761997	758985	0.56	0.53
	762874	753214		
	769820	751208		

Table 38: Intraday and interday precision of Pyridoxamine dihydrochloride

Concentration (µg/ml)	Peak area		% RSD	
	Intraday	Interday	Intraday	Interday
10	273347	271245	0.29	0.22
	274852	270236		
	273654	270148		
15	365260	367854	0.57	1.09
	361245	360214		
	364153	362148		

Table 39: Intraday and interday precision of Resveratrol

Concentration (µg/ml)	Peak area		% RSD	
	Intraday	Interday	Intraday	Interday
10	155132	154726	0.98	1.32
	158642	158742		
	152485	155874		
15	200955	218752	0.94	1.24
	210584	214754		
	214820	213654		

Repeatability:

Repeatability of injection was determined by injecting standard solutions (10µg/ml) of BEN and (5µg/ml) of PYR and RES for six times, noted peak areas and % RSD was calculated (table 40, 41 and 42).

Table 40: Repeatability injection of Benfotiamine

Concentration (µg/ml)	Repeatability	% RSD
10	276467	1.10
	278562	
	274856	
	276984	
	278102	
	270254	

Table 41: Repeatability injection of Pyridoxamine dihydrochloride

Concentration (µg/ml)	Repeatability	% RSD
5	172106	0.18
	173258	
	174852	
	170235	
	174714	
	179680	

Table 42: Repeatability injection of Resveratrol

Concentration (µg/ml)	Repeatability	% RSD
5	75019	0.20
	75128	
	75365	
	75054	
	75147	
	75365	

Limit of detection and limit of quantification (LOD and LOQ):

The LOD and LOQ were found to be respectively in shown, (table 43)

Table 43: LOD and LOQ

Benfotiamine		Pyridoxamine dihydrochloride		Resveratrol	
LOD	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**
0.2µg/ml	0.7µg/ml	0.3µg/ml	0.9 µg/ml	0.3µg/ml	1µg/ml

Specificity:

There were no additional peaks observed while injecting solvents or mobile phase alone. The peak purity index of standard BEN 0.9998, PYR 0.9979 and RES 0.9998.

Robustness:

In order to demonstrate the robustness of the method, the following optimized conditions were slightly changed.

± 0.1ml flow rate

± 0.5% organic solvent

± 0.1 P^H

The responses for these changed chromatographic parameters were almost same for the fixed chromatographic parameters and hence the developed method was said to be robustness.

Stability of solution:

The solution under room temperature was stable for 24 hours. (Table 44)

Table 44: Stability of solution

Hours	Benfotiamine		Pyridoxamine		Resveratrol	
	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
0 hrs	20	570535	10	270137	10	159877
6 hrs	20	577376	10	273347	10	155132
24 hrs	20	567586	10	266242	10	148883

System suitability studies:

The system suitability parameters like peak area, tailing factor, theoretical plate count, resolution, and retention time were calculated from the standard chromatogram (table 45).

Table 45: System suitability studies

Drug	Theoretical plate(N)	Retention time(min)	Tailing factor	Resolution
Benfotiamine	3574.368	4.290	1.36	-
Pyridoxamine	3141.052	2.195	1.40	4.790
Resveratrol	3220.258	20.589	1.18	14.451

Analysis of formulation

Twenty tablets each containing 50mg of BEN, 25mg of PYR and RES was taken for the study and average weight was determined. Quantity equivalent to 20mg of BEN, 10mg of PYR and RES was weight and transferred to 10ml standard flask and it was dissolved and made up to volume with methanol. Further dilutions were made and injected. The chromatogram is shown in fig 45. The result of an analysis of formulation is shown in table 46.

Fig 45: Chromatogram of BEN, PYR and RES equivalent to 10µg/ml

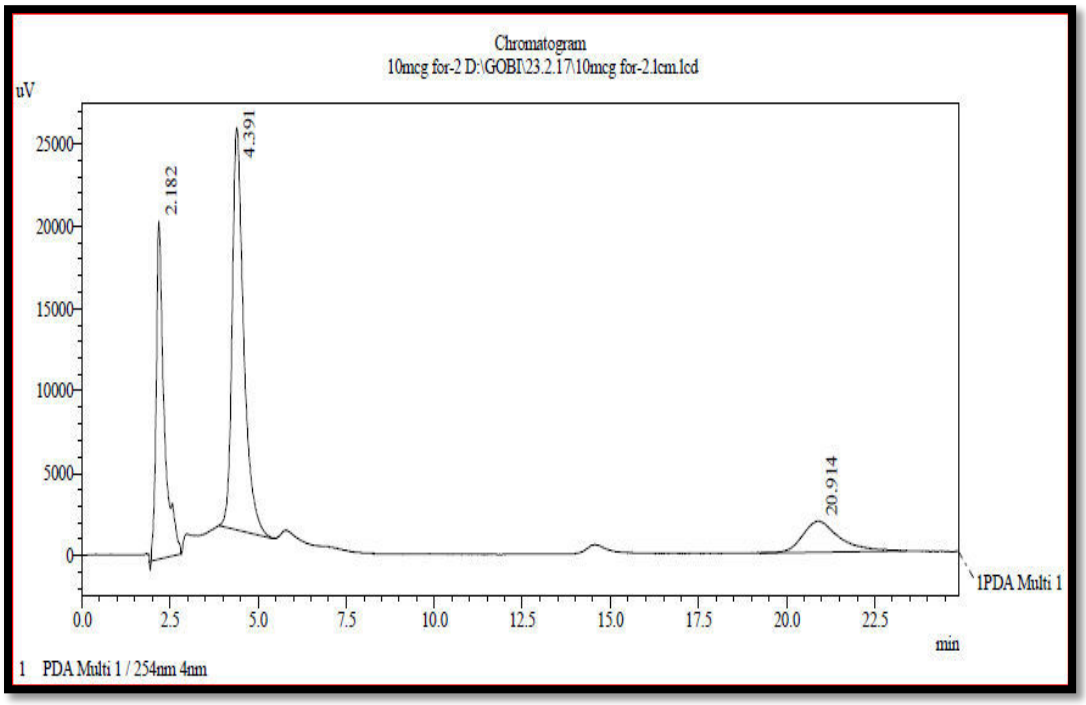


Table 46: Analysis of formulation

Drug	Amount of drug/tab		% label claim	% RSD	SEM
Ageless	Label	Found			
Benfotiamine	50mg	50.5mg	101%	0.18	0.16
Pyridoxamine dihydrochloride	25mg	24.2mg	96.8%	0.58	0.15
Resveratrol	25mg	24.8mg	99.2%	0.38	0.13

***Average of six observation**

Recovery study:

Recovery studies were carried out at 50% and 100% levels. The percentage recovery and % RSD of the results were calculated, table 47. The high % recovery values shows accuracy of the method.

Table 47: Recovery study

% Level	Drug	% Recovery	% RSD	SEM
50	BEN	99.61%	0.84	0.21
	PYR	98.23%	0.47	0.14
	RES	99.47%	0.36	0.23
100	BEN	102.21%	0.96	0.13
	PYR	101.01%	1.14	0.26
	RES	103.24%	0.54	0.14

STATISTICAL ANALYSIS

To ascertain the result obtain in the four methods developed, the statistical analysis was carried out.

Unpaired‘t’ test

Accuracy of UV method:

The % RSD values of the UV method are shown in table 48

Table 48: % RSD of accuracy

Parameter	% RSD
UV	99.00
	98.42
	99.32
	99.41
	99.02
	97.02

These values were evaluated on statistical tool graphpad instant. The calculated P < 0.05 values were found to be 0.5316 which is greater than P value so consider to not significant.

Accuracy of HPTLC method:

The % RSD values of the HPTLC method are shown in table 49.

Table 49: % RSD of Accuracy

Parameter	% RSD
HPTLC	98.54
	99.02
	98.14
	97.54
	98.56
	98.75

These values were evaluated on statistical tool graphpad instant. The calculated P < 0.05 values were found to be 0.0942 which is greater than P value so consider to not significant.

The unpaired‘t’ test carried out for the precision that all the methods are equally precise.

Paired‘t’ test

Comparison of accuracy of existing UV method with developed UV method. The % RSD values of the two methods shown in table 50.

Table 50: % RSD of accuracy

UV	Existing UV
99.00	100.10
98.42	99.97
99.32	98.27
99.41	98.02
99.02	99.91
97.02	100.27

These values were evaluated on statistical tool graphpad instant. The calculated $P < 0.05$ values were found to be 0.2149 which is greater than P value so consider to not significant.

ANOVA

One way analysis of variance (ANOVA) test was applied on precision (intraday and interday precision) studies of the UV and HPTLC methods for Benfotiamine.

Precision of assay:

Intraday precision:

The % RSD values of three methods are shown in table 51.

Table 51: % RSD of intraday precision

UV	HPTLC
0.29	0.58
0.47	1.27
0.97	0.97
0.84	0.74
0.79	0.86
0.68	0.69

These values were evaluated on statistical tool graphpad instant. The calculated $P < 0.05$ values were found to be 0.2207 which is greater than P value so consider to not significant.

Interday precision:

The % RSD values are shown in table 52.

Table 52: % RSD of interday precision

UV	HPTLC
0.09	0.38
0.37	1.24
0.98	0.91
0.83	0.89
1.72	0.68
0.76	0.79

The values were evaluated on statistical tool graphpad instant. The calculated P < 0.05 values were found to be 0.2501 which is greater than P value so consider to not significant.

Table 53: statistical comparison

Parameter	Intraday			Interday		
	Mean difference	't' value	'p' value	Mean difference	't' value	'p' value
UV Vs HPTLC	0.11	7.27	>0.05	0.018	6.54	>0.05

Statistical studies were carried out for HPLC and HPTLC method development of Benfotiamine in combination with Pyridoxamine dihydrochloride and Resveratrol.

Precision of assay for benfotiamine (intraday precision)

The % RSD values of the two methods are shown in table 54.

Table 54: % RSD of intraday precision

HPTLC	HPLC
0.58	0.16
1.27	0.97
0.92	1.97
0.74	0.82
0.86	0.78
0.69	0.62

These values were evaluated on statistical tool graphpad instant. The calculated P < 0.05 values were found to be 0.1697 which is greater than P value so consider to not significant.

Precision of assay for benfotiamine (interday precision)

The % RSD values are shown in table 55.

Table 55: % RSD of interday precision

HPTLC	HPLC
0.38	0.06
1.24	0.93
0.91	1.12
0.89	1.45
0.68	0.76
0.79	0.81

These values were evaluated on statistical tool graphpad instant. The calculated P < 0.05 values were found to be 0.2945 which is greater than P value so consider to not significant.

Table 56: Statistical comparison

Parameter	Mean difference		't' value		'p' value
	Intraday	Interday	Intraday	Interday	
HPTLC Vs HPLC	-0.0350	0.040	0.8476	0.7802	>0.05

Precision of assay for Pyridoxamine dihydrochloride (intraday precision)

The % RSD values of the two methods shown in table 57.

Table 57: % RSD of intraday precision

HPTLC	HPLC
0.62	0.29
0.64	0.57
0.72	0.71
1.02	0.82
0.68	0.62
0.47	0.47

These values were evaluated on statistical tool graphpad instant. The calculated $P < 0.05$ values were found to be 0.2980 which is greater than P value so consider to not significant.

Precision of assay for Pyridoxamine dihydrochloride (interday precision)

The % RSD values are shown in table 58.

Table 58: % RSD of interday precision

HPTLC	HPLC
0.62	0.22
1.44	1.09
0.76	0.94
0.51	0.82
0.49	0.76
0.91	0.54

These values were evaluated on statistical tool graphpad instant. The calculated P < 0.05 values were found to be 0.4245 which is greater than P value so consider to not significant.

Table 59: Statistical comparison

Parameter	Mean difference		't' value		'p' value
	Intraday	Interday	Intraday	Interday	
HPTLC Vs HPLC	-0.036	0.060	0.8670	0.6888	>0.05

Precision of assay for Resveratrol (intraday precision)

The % RSD values of the two methods shown in table 60

Table 60: % RSD of intraday precision

HPTLC	HPLC
1.44	0.98
1.94	0.94
0.97	0.49
0.25	1.21
0.37	0.80
0.72	0.79

These values were evaluated on statistical tool graphpad instant. The calculated P < 0.05 values were found to be 0.2763 which is greater than P value so consider to not significant.

Precision of assay for Resveratrol (interday precision)

The % RSD values are shown in table 61

Table 61: % RSD of interday precision

HPTLC	HPLC
0.94	1.32
1.32	1.24
0.85	0.69
0.71	0.35
0.35	0.47
0.55	0.78

These values were evaluated on statistical tool graphpad instant. The calculated P < 0.05 values were found to be 0.1950 which is greater than P value so consider to not significant.

Table 62: Statistical comparison

Parameter	Mean difference		't' value		'p' value
	Intraday	Interday	Intraday	Interday	
HPTLC Vs HPLC	0.080	0.021	0.7934	0.8531	>0.05

The paired't' test carried out for the precision that all the methods are equally precise.

SUMMARY

In current research work newer analytical methods were developed and validated for benfotiamine in single dosage form and its combination with pyridoxamine dihydrochloride and resveratrol. An UV spectroscopy and stability indicating HPTLC methods were developed for the quantification of benfotiamine in single dosage form and for the estimation of benfotiamine in combined dosage form two chromatographic methods viz a HPTLC and HPLC were developed.

Analytical methods for benfotiamine in single dosage form

The development of UV spectrophotometric method involved scanning of the standard and sample solutions in the UV range using UV visible spectrophotometer. Benfotiamine showed maximum absorbance at 240nm. The method was validated for linearity, precision, accuracy and stability. An overly of different concentrations of benfotiamine was noted with absorbance at λmax. The linear concentration was 4-24µg/ml and the correlation value was 0.999. The percentage RSD values of repeatability, interday and intraday were found to be < 2, which proves precision of the method. The drug solution was found to be stable up to 48hrs at room temperature. The method was the applied to estimation of Benfotiamine bulk drug and tablet formulation.

For the estimation of Benfotiamine by HPTLC method, the sample solutions were applied on silica gel $G_{60}F_{254}$ TLC plates and the chromatogram was developed using mobile phase comprising of Glacial acetic acid : Methanol : Triethylamine (9: 1% v/v, 2drops) and detection was carried out at 275 nm using densitometer. The R_f value of Benfotiamine was 0.29±0.02, which showed linearity range from 0.1 – 0.6µg/spot and correlation coefficient 0.998. The percentage RSD values of repeatability, intraday and interday were found to be < 2. It proves precision of the method.

HPTLC method was applied for accelerated stability studies of Benfotiamine in bulk drug and tablet formulation for acid, alkali and oxidative degradation. The degradation study indicated benfotiamine is more susceptible to acid hydrolysis than other conditions. There were no additional peak observed when treated with all the degradation conditions, but reduction in peak area was found. The tablet placebo was prepared and subjected to degradation studies as like formulation, to know if any interferences due to this. It was observed that there was no additional peak at the R_f value of benfotiamine due to excipients.

The statistical analysis was applied to the assay results obtained by two newly developed methods (UV and HPTLC). The results of an unpaired't' test conducted is shown in table below.

Parameter	**UV**	**HPTLC**
Mean (assay)	98.63	98.42
Number of points	6	6
SD	0.982	0.520
SEM	0.364	0.212
Median	99.010	98.550
P	0.5316	
F	2.937	

For the statistical comparison of the developed UV method and existing UV method [13] for benfotiamine a paired 't' test was conducted the results as shown in table below.

Parameter	**UV**	**UV**
Mean (assay)	98.69	99.42
Number of points	6	6
SD	0.892	1.001
SEM	0.364	0.408
Median	99.10	99.940
P		0.2149
F	1.258	

The results proved that there is no statistical significant difference among two developed methods for estimation of benfotiamine.

One way ANOVA was carried out for the intraday and interday precision of UV and HPTLC method for benfotiamine estimation from tablets. The average mean difference was 0.11, t- value, 7.27 and p- value was >0.05 for intraday precision. For interday precision was mean difference 0.018, t- value 6.54 and p-value was>0.05.

Analytical method for benfotiamine in combination with pyridoxamine dihydrochloride and resveratrol

A newer HPTLC method was developed for estimation of benfotiamine in combination of pyridoxamine dihydrochloride and resveratrol combined dosage form. The sample solutions were applied TLC plates and the chromatogram was developed using mobile phase separation of Glacial acetic acid: petroleum ether: Acetone: Triethylamine: Tetra butyl ammonium bromide (5.5:1.5:3% v/v, 5drops, and 2drops) and detection was carried out a 285nm. The linearity range from benfotiamine 200-1200ng/spot, pyridoxamine dihydrochloride 100-600ng/spot and respectively for benfotiamine, pyridoxamine dihydrochloride and resveratrol. The method was successfully applied to the formulation containing these three drugs.

A new high performance liquid chromatographic method was developed for determination of benfotiamine in combination with pyridoxamine dihydrochloride and resveratrol in tablet formulation. The chromatographic separation was performed on stationary phase of Lichrospher®100 RP – 18e (5µm) and mobile phase of 10mM KH_2PO_4 (pH- 4.9): Methanol + Acetonitrile and solvent ratio of 70: 15+15 %v/v, flow rate: 1.0ml/min. The detection wavelength was at 254nm.

The method was validated for linearity, precision, stability and robustness. The linear concentration benfotiamine was 10-50µg/ml, pyridoxamine dihydrochloride 5-25µg/ml and resveratrol 5-25µg/ml. The correlation value was 0.999, 0.997 and 0.999 respectively for benfotiamine, pyridoxamine dihydrochloride and resveratrol. The percentage RSD values of repeatability, interday and intraday precision were found to be < 2. It proves precision of the method. The solution was stable up to 36 hours at room temperature. The method was robust for very minor change in the changed chromatographic parameters.

A paired't' test was conducted to test the precision of the two methods for every single drug present in combined dosage form. The results of paired t-test are benfotiamine in shown table below.

Parameter	**HPTLC**		**HPLC**	
	Intraday	**Interday**	**Intraday**	**Interday**
Mean (precision)	0.84	0.88	0.81	0.85
Number of points	6	6	6	6
SD	0.241	0.599	0.284	0.463
SEM	0.098	0.244	0.116	0.189
Median	0.800	0.800		0.368
P	0.063		0.060	
T	0.2024		0.2945	

The statistical comparison of precision pyridoxamine dihydrochloride by paired 't' test in shown table below.

Parameter	**HPTLC**		**HPLC**	
	Intraday	**Interday**	**Intraday**	**Interday**
Mean (precision)	0.69	0.58	0.78	0.72
Number of points	6	6	6	6
SD	0.182	0.185	0.356	0.309
SEM	0.074	0.075	0.145	0.126
Median	0.660	0.595	0.690	0.790
P	0.08		0.17	
T	2.217		0.424	

The paired t-test results of resveratrol is shown table below.

Parameter	HPTLC		HPLC	
	Intraday	**Interday**	**Intraday**	**Interday**
Mean (precision)	0.94	0.86	0.78	0.80
Number of points	6	6	6	6
SD	0.648	0.240	0.336	0.396
SEM	0.264	0.098	0.137	0.162
Median	0.845	0.870	0.780	0.735
P	0.793		0.853	
T	0.276		0.195	

The results proved that there is no statistical significant difference among two and three developed methods for estimation of benfotiamine and it combination with pyridoxamine dihydrochloride and resveratrol.

CONCLUSION

The UV, HPTLC and HPLC methods developed were validated as per ICH guidelines and found to be specific, accurate and precise. The results of all the methods were ascertained by statistical analysis.

Among the two analytical methods developed, HPTLC is more sensitive technique, whereas UV is a simpler technique for the estimation of Benfotiamine in single dosage form. The HPTLC is more sensitive than HPLC method for the analysis of Benfotiamine in combination with Pyridoxamine dihydrochloride and Resveratrol. However HPLC offers a more reliable quantification than HPTLC for combination.

All the four methods were successfully employed to analyse benfotiamine from single dosage form or in combination with Pyridoxamine dihydrochloride and Resveratrol. They would serve as good analytical tools for the quality control and research involving Benfotiamine and its combined dosage form with Pyridoxamine dihydrochloride and Resveratrol.

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